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13. ABSTRACT (Maximum 200 Words)

The present research has developed a non-viral gene targeting technology, whereby the effects of a neurotoxin on the brain can be reversed shortly after the intravenous injection of a therapeutic gene medicine without the use of viral vectors. The brain gene targeting technology developed in this work creates an "artificial virus" which is comprised of non-immunogenic lipids and proteins, wherein the therapeutic gene is packaged in the interior of the gene delivery vehicle, which is called a pegylated immunoliposome (PIL). The PIL carrying the gene is a 85 nm "stealth" nanocontainer, which is relatively invisible to the body's reticuloendothelial system, which normally removes nanocontainers from the blood. The surface of the nanocontainer is studded with a receptor-specific monoclonal antibody (MAb). This MAb acts as a moleculr Trojan horse, and triggers the transport of the stealth nanocontainer across the 2 biologicial membrane barriers that separate the blood from the interior of brain cells. These barriers are the brain microvascular endothelial wall, which forms the blood-brain barrier in vivo, and the brain cell plasma membrane. Both barriers express the transferrin receptor, and the anti-receptor MAb enables the PIL to cross the membrane barriers via normal physiological transport processes usually used for endogeneous ligands such as transferrin. With this approach non-viral, non-invasive gene therapy of the brain is now possible.

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Table of Contents

INTRODUCTION

Neurotoxins can cause serious derangements in brain biochemistry that can compromise the cognitive and motor function of the individual. In the present studies an animal model of neurotoxin exposure is used, wherein the neurotoxin, 6-hyrdoxydopamine, is injected into a specific region of the rat brain called the medial forebrain bundle, followed approximately 4 weeks later by a biochemical picture resembling Parkinson's disease (PD). On the side of the brain where the neurotoxin is injected, there is a 90% reduction in the level of a key enzyme, tyrosine hydroxylase (TH), which is a rate-limiting enzyme involved in dopamine production. Dopamine is the neurotransmitter that is deficient in PD. One way that brain TH levels can be restored in conditions such as PD is through gene therapy, wherein the TH gene is given to the individual afflicted with PD. However, with the conventional approach to gene therapy of the brain, there are two serious problems. First, virtually all present-day approaches use viral vectors to carry the gene to brain cells. However, these viral vectors are either highly inflammatory (such as adenovirus or herpes simplex virus) or stably alter the host genome in a random way (retrovirus, adeno-associated virus), which can lead to insertional mutagenesis and cancer. These viruses do not enter the brain from blood, because they do not cross the blood-brain barrier (BBB). This creates the second problem with present-day approaches to gene therapy, which is the viral vector is administered to the brain by craniotomy and drilling a hole in the head. However, this only distributes the virus to a tiny region of the brain at the tip of the injection needle. What is needed is a non-invasive, non-viral form of brain gene therapy wherein the therapeutic gene can be administered intravenously without viral vectors followed by widespread expression of the exogenous gene throughout the brain. This is the goal of the present research.

The present research uses a completely new form of brain gene targeting technology which uses a novel gene delivery vehicle called pegylated immunoliposomes (PIL). PILs are comprised of non-immunogenic lipids and proteins, wherein the therapeutic gene is packaged within the interior of the gene delivery vehicle, which is called a pegylated immunoliposome (PIL). The PIL carrying the gene is an 85 nm "stealth" nano-container, which is relatively invisible to the body's reticuloendothelial system, that normally removes nano-containers from the blood. This stealth effect is created by conjugating approximately 2000 strands of 2000 Dalton polyethylene glycol (PEG) to the surface of the liposome carrying the gene inside. Approximately 1-2% of the tips of the PEG strands are studded with receptor-specific monoclonal antibodies (MAb). This MAb is a targeting ligand and acts as a molecular Trojan horse, which triggers the transport of the stealth nano-container across the two biological membrane barriers which separate the blood from the interior of brain cells: the brain microvascular endothelial wall, which forms the blood-brain barrier (BBB) in vivo, and the brain cell plasma membrane (BCM). Both the BBB and the BCM express a targeted receptor, in this case, the transferrin receptor (TfR), and the anti-TfR MAb enables the PIL to cross the membrane barriers via normal physiological transport processes which are usually used for endogenous ligands such as transferrin. With this approach, non-viral gene therapy, non-invasive gene therapy of the brain is now possible.

The TH expression plasmid is encapsulated in the interior of the 85 nm PIL which is targeted to rat brain with the OX26 murine MAb to the rat TfR. The TfRMAb-PIL carrying the plasmid DNA is injected intravenously in rats at a dose of 1-10 μg plasmid DNA per adult rat. These rats all have drug-confirmed experimental PD, owing to the intracerebral injection of the 6-hydroxydopamine neurotoxin into the brain four weeks earlier. The goal is to normalize the striatal TH activity based on both brain biochemistry assays, immunocytochemistry assays, and pharmacologic behavioral testing.

BODY

Original Statement of Work (SOW). The original statement of work outlined experiments in 5 areas:
(1) formulation of PILs (01 year)
(2) single dose efficacy study with SV40 promoter (01-02 years)
(3) toxicity study with focus on brain inflammatory response (02-03 years)
(4) single dose efficacy study with glial fibrillary acidic protein (GFAP) gene promoter (02-03 years)
(5) multi-dose efficacy study (03 year)

Progress toward these goals has been achieved on time. In prior Progress Reports for the 01 and 02 years, we demonstrated completion of the work in (1), (2), and (3) of the SOW. In the past 03 year, we completed the work in (4) of the SOW. We have requested and received a no-cost time extension to complete (5) of the SOW during an 04 year.

Progress in 03 Year. In the past 03 year, we published the toxicity study (**Appendix 1** attached). In this study, rats were treated with 6 weeks of multi-dosing (weekly dosing for 6 weeks), and no toxicity was found, as reported last year.

In the past 03 year, we published our study on the treatment of experimental PD with a TH expression plasmid under the influence of the brain specific promoter taken from the 5'-flanking sequence of the human glial fibrillary acidic protein (GFAP) gene. This work is described in **Appendix 2.** This work was described in part a year ago. Since then, we performed real time polymerase chain reaction (PCR) studies to verify expression of the TH gene following intravenous delivery with PILs, and the real time PCR results are given in Appendix 2..

In the past 03 year, we completed a study showing the organ-specific pattern of gene expression in the eye and the brain of the mouse when the SV40 or GFAP promoter was replaced with a rhodopsin promoter (**Appendix 3**).

KEY RESEARCH ACCOMPLISHMENTS

The following manuscripts were produced in the current 03 year:

- Zhang, Y., Boado, R.J., and Pardridge, W.M. (2003): Absence of toxicity of chronic weekly intravenous gene therapy with pegylated immunoliposomes. Pharm. Res., 20: 1770-1785. **(APPENDIX 1)** This work is important because it shows that the PILs carrying a therapeutic gene can be chronically administered without any toxic effects. This work was submitted a year ago as a manuscript submitted for publication, and the final reprint is now attached.

- Zhang, Y., Schlachetzki, F., Zhang, Y., Boado, R.J., and Pardridge, W.M. (2004): Normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental Parkinsonism with intravenous non-viral gene therapy and a brain –specific promoter. Human Gene Therapy, 15: 339-350. **(APPENDIX 2)** This work is important because it shows that ectopic expression of the TH gene in non-brain organs is eliminated with the replacement of the widely read SV40 promoter with the brain-specific gene promoter taken from the 5'-flanking sequence of the human GFAP gene. This work was submitted a year ago as a manuscript submitted for publication, and the final reprint is now attached.

- Zhu, C., Zhang, Y., Zhang, Y.F., Li, J.Y., Boado, R.J., and Pardridge, W.M. (2004): Organ specific expression of LacZ gene controlled by the opsin promoter after intravenous gene administration in adult mice. J. Gene Med., in press. This work shows that the rhodopsin promoter, like the GFAP promoter, enables brain-specific gene expression of an exogenous gene. (**APPENDIX 3**).

- Pardridge, W.M. (2003): Gene targeting in vivo with pegylated immunoliposomes. Methods in Enzymology (Liposomes, Part C), 373: 507-528. This review article describes in detail the methods for production of PILs.

- Schlachetzki, F., Zhang, Y., Boado, R. J., and Pardridge, W.M. (2004): Gene therapy of the brain: the transvascular approach. Neurology, 62: 1275-1281. This review article describes how this new form of brain gene delivery can be used to treat many disorders of the brain without viruses and with a route of administration no more invasive than a simple intravenous injection.

REPORTABLE OUTCOMES

(1) Manuscripts: listed below in Publications.

(2) Plasmids developed: tyrosine hydroxylase expression plasmids driven by either the SV40 or the GFAP promoter were produced as described in Appendix 2.

CONCLUSIONS

The US Army support of this work has led to the development of a transformational technology that revolutionizes our approach to gene therapy of the brain and to gene therapy of brain neurotoxin exposure. Unlike the conventional approach to brain gene therapy, we achieve the desired pharmacological effect without viruses and without craniotomy. The use of viral vectors will probably never be widely used in gene therapy in humans, owing to their toxic effects. The use of craniotomy for delivering genes to the brain is problematic because the gene is only delivered to a tiny area of the brain. Moreover, craniotomy-based gene therapy of the brain in soldiers in the field is virtually impossible. We have created a form of brain gene therapy that could be administered to soldiers in the field.

This approach to gene therapy enables adult transgenics in 24 hours.

PUBLICATIONS FUNDED BY THIS WORK TO DATE:

1. Shi, N., Zhang, Y., Boado, R.J., Zhu, C., and Pardridge, W.M. (2001): Brain-specific expression of an exogenous gene following intravenous adminstration. Proc. Natl. Acad. Sci. U.S.A., 98: 12754-12759. **(Reviewed in *Nature Reviews-Neuroscience* 2: 852-853, December, 2001.)**

2. Pardridge, W.M. (2002): Drug and gene targeting to the brain with molecular Trojan horses. Nature Reviews-Drug Discovery, 1: 131-139.

3. Pardridge, W.M. (2002): Drug and gene delivery to the brain: the vascular route. Neuron, 36: 555-558. **(6th most frequently downloaded journal article in *Neuron*)**

4. Zhang, Y., Zhu, C., and Pardridge, W.M. (2002): Antisense gene therapy of brain cancer with an artificial virus gene delivery system. Mol. Ther., 6: 67-72.

5. Zhang, Y., Boado, R.J., and Pardridge, W.M. (2003): Marked enhancement in gene expression by targeting the human insulin receptor. J. Gene Med., 5: 157-163.

6. Zhang, Y., Calon, F., Zhu, C., Boado, R.J., and Pardridge, W.M. (2003): Intravenous non-viral gene therapy causes complete normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental Parkinsonism. Hum. Gene Ther. 14: 1-12. **(Journal Cover Illustration)**

7. Zhang, Y., Schlachetzki, F., and Pardridge, W.M. (2003): Global non-viral gene transfer to the primate brain following intravenous administration. Mol. Ther., 7: 11-18. **(Highlighted in 'Inside This Month' of *Molecular Therapy*; reviewed in March 22, 2003, *New Scientist;* reviewed in June, 2003 *Drug Discovery Today*, 8:473-474.)**

8. Zhang, Y., Boado, R.J., and Pardridge, W.M. (2003): Absence of toxicity of chronic weekly intravenous gene therapy with pegylated immunoliposomes. Pharm. Res., 20: 1770-1785. **(INCLUDED AS APPENDIX 1)**

9. Zhang, Y., Schlachetzki, F., Li, J.Y., Boado, R.J., and Pardridge, W.M. (2003): Organ-specific gene expression in the Rhesus monkey eye following intravenous non-viral gene transfer. Mol. Vis., 9: 465-472.

10. Pardridge, W.M. (2003): Gene targeting in vivo with pegylated immunoliposomes. Methods in Enzymology (Liposomes, Part C), 373: 507-528.

11. Zhang, Y., Schlachetzki, F., Zhang, Y., Boado, R.J., and Pardridge, W.M. (2004): Normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental Parkinsonism with intravenous non-viral gene therapy and a brain –specific promoter. Human Gene Therapy, 15: 339-350. **(INCLUDED AS APPENDIX 2)**

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13. Zhu, C., Zhang, Y., Zhang, Y.F., Li, J.Y., Boado, R.J., and Pardridge, W.M. (2004): Organ specific expression of LacZ gene controlled by the opsin promoter after intravenous gene administration in adult mice. J. Gene Med., in press. **(INCLUDED AS APPENDIX 3)**

APPENDICES:

Appendix 1

Zhang, Y., Boado, R.J., and Pardridge, W.M. (2003): Absence of toxicity of chronic weekly intravenous gene therapy with pegylated immunoliposomes. Pharm. Res., 20: 1770-1785.

Appendix 2

Zhang, Y., Schlachetzki, F., Zhang, Y., Boado, R.J., and Pardridge, W.M. (2004): Normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental Parkinsonism with intravenous non-viral gene therapy and a brain –specific promoter. Human Gene Therapy, 15: 339-350.

Appendix 3

Zhu, C., Zhang, Y., Zhang, Y.F., Li, J.Y., Boado, R.J., and Pardridge, W.M. (2004): Organ specific expression of LacZ gene controlled by the opsin promoter after intravenous gene administration in adult mice. J. Gene Med., in press.

Absence of Toxicity of Chronic Weekly Intravenous Gene Therapy with Pegylated Immunoliposomes

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Purpose. Plasmid DNA-based gene therapy involves episomal gene expression and must be given on a chronic basis. Therefore, the purpose of the present study was to examine for toxic side effects of the chronic weekly intravenous administration of plasmid DNA delivered with a nonviral gene transfer method using pegylated immunoliposomes (PIL).

Methods. A 7-kb expression plasmid encoding for rat tyrosine hydroxylase (TH) was encapsulated in PILs targeted with either the murine OX26 monoclonal antibody (MAb) to the rat transferrin receptor (TfR) or with the mouse IgG2a isotype control antibody. Rats were treated with weekly intravenous injections of 5 μg/rat/week of the TH expression plasmid DNA encapsulated in either the TfRMAb-targeted PIL or the mouse IgG2a-targeted PIL for a total period of 6 weeks. A third control group of rats was treated with saline.

Results. The animals treated with either saline, the TfRMAb-PIL, or the mouse IgG2a-PIL had no measurable differences with respect to body weights, 14 serum chemistries, or organ histology of brain, liver, spleen, kidney, heart, or lung. Immunocytochemistry showed no evidence of inflammation in brain. The delivery to brain of the TH expression plasmid was confirmed with Southern blotting.

Conclusions. The PIL nonviral gene transfer method causes no toxic side effects following chronic weekly intravenous administration in rats.

KEY WORDS: gene therapy; brain; liposomes; nonviral gene transfer; inflammation.

INTRODUCTION

An important issue with either viral or nonviral gene delivery systems is organ toxicity associated with the delivery vector (1). In the case of either adenovirus or Herpes simplex virus, the preexisting immunity to these viruses causes an inflammatory reaction (2,3). A single injection of either adenovirus or Herpes simplex virus into the brain causes inflammation leading to demyelination (4,5). More than 90% of the human population has a preexisting immunity to adeno-associated virus (6). Therefore, there is a need to establish nonviral gene transfer technology with minimal toxicity. The principal forms of nonviral gene transfer include the use of complexes of DNA/cationic polymers or the hydrodynamic injection method. Cationic polyplexes have a relatively narrow therapeutic index. A nitrogen/phosphate (N/P) ratio of 6–10 is necessary for gene expression in the lung following the intravenous injection of the cationic polymer/plasmid DNA complexes, whereas an N/P ratio >20 is lethal (7). The hydrodynamic method involves the rapid intravenous injection of a volume of saline greater than the existing blood volume of the animal. This results in transitory right heart failure and hepatic congestion causing a selective expression of plasmid DNA in the liver (8). This gene delivery method results in an increase in liver enzymes, and the mortality with this method can be as high as 40% depending on the salt solution injected (8).

An alternative form of nonviral gene transfer involves the use of pegylated immunoliposomes (PIL). In this formulation, the nonviral plasmid DNA is encapsulated in the interior of an 85-nm liposome that has a net anionic charge (9). The surface of the liposome is pegylated with several thousand strands of 2000-Da polyethyleneglycol (PEG). The pegylated liposome is then targeted to distant sites by conjugating a transporting ligand to the tips of 1–2% of the PEG strands. Peptidomimetic monoclonal antibodies (MAb) to either the transferrin receptor (TfR) or the insulin receptor (IR) have been used to target PILs carrying expression plasmids to distant sites following intravenous injection (9,10). The PILs do not aggregate in saline and have prolonged blood residence times (11). PILs have been administered intravenously to mice on a weekly basis for the treatment of brain cancer (12), and PILs have been given to rats for the treatment of experimental Parkinson's disease (13). PILs targeted with the TfRMAb have been used to deliver nonviral plasmid DNA to brain. Because of the expression of the TfR on both the blood–brain barrier (BBB) and the neuronal plasma membrane, the TfRMAb-targeted PIL delivers the plasmid DNA to brain as well as other organs rich in TfR, such as liver and spleen (9,14). However, to date, there has been no evaluation of the potential toxicity of repeat intravenous administration of PILs.

The purpose of the present study was to examine the potential toxicity of repeat weekly intravenous administration of PIL-encapsulated plasmid DNA that was targeted to tissues in the rat with either the murine OX26 MAb to the rat TfR, or PILs targeted with the corresponding mouse IgG2a isotype control antibody. The plasmid DNA used in the present studies is the clone 877 DNA, which encodes for rat tyrosine hydroxylase (TH), as described previously (13). The delivery of the TH expression plasmid to brain with the TfRMAb-targeted PIL results in a normalization of striatal TH enzyme activity in brain of rats lesioned with a neurotoxin (13). For the present toxicity study, the TH expression plasmid DNA was encapsulated in either the TfRMAb-PIL or the mIgG2a-targeted PIL and was injected weekly for 6 weeks at a dose of 5 μg/rat of PIL-encapsulated plasmid DNA. Body weights of the animals were determined during the treatment period, and at the end of the 6-week treatment, blood was obtained for measurement of 14 parameters of serum chemistry reflecting liver and renal function. Major organs were removed at the end of the treatment period for pathologic analysis. In addition, brain was examined in detail with immunocytochemistry using antibodies to multiple antigens that reflect underlying tissue inflammation. Immunocytochemistry of brain was performed with the mouse OX1 MAb to rat leukocytes, the mouse OX2 MAb to the rat class II multiple histocompatibility complex (MHC) antigen, the mouse OX18

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MAb to the rat class I MHC antigen, the mouse OX35 MAb to the rat lymphocyte CD4 receptor, and the mouse OX42 MAb to the rat macrophage. Finally, the present studies used Southern blotting to confirm distribution of the TH expression plasmid in brain following targeting with the TfRMAb-PIL.

METHODS

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) and didodecyldimethylammonium bromide (DDAB) were purchased from Avanti-Polar Lipids Inc. (Alabaster, AL). Distearoylphosphatidylethanolamine (DSPE)-PEG^{2000} was obtained from Shearwater Polymers (Huntsville, AL), where PEG^{2000} is polyethylene glycol (PEG) of 2000 Daltons. DSPE-PEG^{2000}-maleimide was custom-synthesized by Shearwater Polymers. The LiposoFAST-Basic extruder and polycarbonate filters were from Avestin (Ottawa, Canada). [α-^{32}P]dCTP (3000 Ci/mmol) was from NEN Life Science Products Inc. (Boston, MA). *N*-Succinimidyl[2,3-^{3}H]propionate ([^{3}H]NSP, 101 Ci/mmol), Sepharose CL-4B, and Protein G-Sepharose CL-4B were from Amersham Pharmacia Biotech (Arlington Heights, IL). The nick translation system was purchased from Invitrogen Life Technologies (Carlsbad, CA). Exonuclease III was purchased from Promega (Madison, WI); 2-iminothiolane (Traut's reagent) was obtained from Pierce Chemical Co. (Rockford, IL). Mouse myeloma ascites containing IgG2a (κ) (mIgG2a), pancreatic DNase I with a specific activity of 2000 Kunitz units/mg, horse serum, mouse IgG1 isotype, mouse anti–glia fibrillary acidic protein (GFAP) monoclonal antibody (MAb), and glycerol-gelatin were from Sigma Chemical Co. (St. Louis, MO). The antitransferrin receptor monoclonal antibody (TfRMAb) used in these studies is the murine OX26 MAb to the rat TfR, which is a mouse IgG2a. TfRMAb and mIgG2a were individually purified by protein G affinity chromatography from hybridoma-generated ascites. The biotinylated horse anti-mouse IgG, Vectastain ABC kit, and 3-amino-9-ethylcarbazole (AEC) substrate kit were purchased from Vector Laboratories (Burlingame, CA). Mouse antirat class I multiple histocompatibility complex (MHC) monoclonal antibody (OX18), mouse antirat leukocyte CD45 (OX-1), mouse antirat lymphocyte CD4 (OX-35), mouse antirat class II MHC (OX-6), and mouse antirat macrophage CD11b (OX42) were purchased from Serotec (Raleigh, NC). Optimal cutting temperature (O.C.T.) compound (Tissue-Tek) was purchased from Sakura FineTek (Torrance, CA). Adult male Sprague-Dawley rats (weighing from 180–220 g) were obtained from Harlan Breeders (Indianapolis, IN).

Plasmid DNA Preparation and Radiolabeling

The tyrosine hydroxylase expression plasmid, driven by the SV40 promotor and designated clone 877, was constructed as described previously (13). Clone 877 plasmid DNA was purified from *E. coli* with the Plasmid Maxi Kit and desalted per the manufacturer's instructions (Qiagen, Chatsworth, CA). The size of the DNA was confirmed by 0.8% agarose gel electrophoresis. DNA was labeled with [^{32}P]dCTP using nick translation. The specific activity of [^{32}P]DNA was 15–20 μCi/μg. The trichloroacetic acid precipitability was 99%.

PEGylated Liposome Synthesis and Plasmid Encapsulation

POPC (18.8 μmol), DDAB (0.6 μmol), DSPE-PEG^{2000} (0.6 μmol), and DSPE-PEG^{2000}-maleimide (0.2 μmol) were dissolved in chloroform, followed by evaporation, as described previously (14). The lipids were dispersed in 0.2 ml of 0.05 M Tris-HCl buffer (pH 7.0) and vortexed for 1 min, followed by 2 min of bath sonication. Supercoiled DNA (200 μg) and 1 μCi of [^{32}P]DNA were added to the lipids. The dispersion was frozen in ethanol/dry ice for 5 min and thawed at room temperature for 25 min, and this freeze–thaw cycle was repeated five times to produce large vesicles with the DNA loosely entrapped inside. The large vesicles were converted into small (85-nm-diameter) liposomes by extrusion. The liposome dispersion was diluted to a lipid concentration of 40 mM, followed by extrusion five times each through two stacks each of 200- and 100-nm pore size polycarbonate membranes with a hand-held LiposoFAST-Basic extruder as described previously (11). The mean vesicle diameters were determined by quasielastic light scattering using a Microtrac Ultrafine Particle Analyzer (Leeds-Northrup, St. Petersburg, FL) as described previously (11).

The plasmid adsorbed to the exterior of the liposomes was removed by nuclease digestion, and 6 U of pancreatic endonuclease I and 33 U of exonuclease III were added in 5 mM $MgCl_2$ to the liposome/DNA mixture after extrusion. After incubation at 37°C for 1 h, the reaction was stopped by adding 20 mM EDTA. The nuclease digestion removed any exteriorized plasmid DNA, as demonstrated by agarose gel electrophoresis and ethidium bromide staining of aliquots taken before and after nuclease treatment, as described previously (11). The formulation before antibody conjugation is designated a pegylated liposome (PL), and the formulation after antibody conjugation is called a pegylated immunoliposome (PIL).

MAb Conjugation to the PEGylated Liposome Encapsulated with DNA

TfRMAb or mIgG2a was thiolated and individually conjugated to the maleimide moiety of the PEGylated liposome to produce the PIL with the desired receptor specificity. PIL conjugated with the OX26 MAb is designated TfRMAb-PIL, and PIL conjugated with the mIgG2a isotype control is designated mIgG2a-PIL. Either MAb or mIgG2a was radiolabeled with [^{3}H]NSP as described previously (15). [^{3}H]MAb had a specific activity of >0.11 μCi/μg and a TCA precipitability of >97%. The MAb (3.0 mg, 20 nmol) was thiolated with 40:1 molar excess of 2-iminothiolane (Traut's reagent), as described previously (15). The thiolated MAb, which contained a trace amount of ^{3}H-labeled MAb, was conjugated overnight to the PEGylated liposome with encapsulated plasmid DNA containing a trace amount of [^{32}P]DNA. The unconjugated MAb and the oligonucleotides produced by nuclease treatment were separated from the PIL by Sepharose CL-4B column chromatography as described previously (11). The number of MAb molecules conjugated per liposome was calculated from the total ^{3}H-labeled MAb radioactivity in the liposome pool and the specific activity of the labeled MAb, assuming 100,000 lipid molecules per liposome, as described previously (15). The average number of MAb molecules conjugated per liposome was 57 ± 12 (mean ± SD, n = 4 syntheses). The final percentage entrapment of 200 μg of plasmid DNA in the liposome preparation was computed from the ^{32}P radioactivity and was 30 ± 2% (mean ± SD, n = 4 syntheses), or 60 μg of plasmid DNA.

Chronic Intravenous Administration of PIL-Encapsulated DNA

Adult male Sprague-Dawley rats weighing 200–220 g were anesthetized with ketamine (50 mg/kg) and xylazine (4 mg/kg) intraperitoneally. Animals were divided into three groups. PIL or saline was injected i.v. via femoral vein with a 30-g needle. The first group was injected with TfRMAb-PIL carrying clone 877 plasmid DNA at a dose of 5 μg per rat. The second group was injected with mIgG2a-PIL carrying clone 877 plasmid DNA at a dose of 5 μg per rat. The third group was injected with saline. The average intravenous injection volume for all treatments was 300 μL. These intravenous treatments were given once a week for 6 consecutive weeks. Each week before injection, the body weight for each rat was measured. At 3 days following the sixth injection, the rats were anesthetized, and blood was collected from the vena cava. Serum was stored at –20°C for serum chemistry measurements by autoanalyzer in the UCLA Medical Center Clinical Laboratory. The rats were then sacrificed, and organs were removed for immunocytochemistry.

Immunocytochemistry

Immunocytochemistry was performed by the avidin–biotin complex (ABC) immunoperoxidase method (Vector Laboratories). Brains were removed immediately after sacrifice, and cut into three sagittal slabs. One slab was immersion fixed in cold 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) for 24 h at 4°C. The second slab was fixed in cold 100% methanol for 24 h at –20°C. These slabs were cryoprotected in 20% sucrose in 0.1 M phosphate-buffered water, pH 7.4 (PBW), for 24 h at 4°C, and 30% sucrose in PBW for 24 h at 4°C. Brains were embedded in O.C.T. medium and frozen in dry ice powder. Frozen sections (20 μm) of rat brain were cut on a Mikron HM505E cryostat. Endogenous peroxidase was blocked with 0.3% H_2O_2 in 0.3% horse serum–phosphate-buffered saline (PBS) for 30 min. Nonspecific binding of proteins was blocked with 10% horse serum in PBS for 30 min. Sections were then incubated in primary antibodies overnight at 4°C. Based on either results provided by the manufacturer or on pilot studies, the fixative (methanol or paraformaldehyde) was chosen to preserve the target antigenicity in the fixed tissue. For methanol-fixed brain sections, OX1 (5 μg/ml), OX18 (5 μg/ml), or OX35 (5 μg/ml) was used as the primary antibody; for paraformaldehyde-fixed brain sections, OX6 (5 μg/ml), OX42 (5 μg/ml), or mouse anti-GFAP MAb (1 μg/ml) was used as the primary antibody. Identical concentrations of isotype control antibody were also used as primary antibody. Mouse IgG1 was used as the isotype control antibody for OX18, OX1, OX6, and GFAP, and mouse IgG2a was used as the isotype control antibody for OX35 and OX42. After incubation and wash in PBS, sections were incubated in biotinylated horse antimouse IgG for 30 min. After development in AEC, sections were mounted with glycerol-gelatin and examined by light microscopy.

Hematoxylin and Eosin Staining of Rat Organs

The third sagittal slab of brain, as well as liver, spleen, kidney, heart, and lung of each rat were removed and immersion fixed in 10% formalin in 0.1 M phosphate buffer for 48 h at 4°C. The fixed organs were embedded in paraffin and stained with hematoxylin and eosin and examined by light microscopy.

Southern Blotting

Plasmid DNA was isolated with the Hirt procedure (16) from rat brain 3 days following the intravenous injection of saline, clone 877 encapsulated in TfRMAb-PIL, or clone 877 encapsulated in mIgG2a-PIL. Rat brain (100 mg) was homogenized in 2 ml lysis buffer (20 mM Tris pH 7.5, 10 mM EDTA, 1% SDS) containing 15 μg/ml DNase-free RNase A using a Polytron PT-MR 3000 homogenizer (Littau, Switzerland) at full speed for 10 s Samples were incubated for 30 min at 37°C. Proteinase K was added to a final concentration of 1 mg/ml and samples incubated for 2 h at 37°C. Nuclear DNA was precipitated overnight at 4°C in the presence of 1.1 M NaCl. Samples were centrifuged at 14,000 rpm and 4°C for 30 min. Supernatants were extracted with phenol:chloroform, and plasmid DNA precipitated with ethanol in the presence of 10 μg glycogen carrier. Aliquots of precipitated material were resolved by gel electrophoresis in 0.8% agarose and blotted onto a GeneScreen Plus membrane (14). To prevent hybridization with the endogenous rat TH genomic DNA, membranes were hybridized with [^{32}P]pGL2 clone 734 (17), which contains the clone 877 backbone but without the rat TH cDNA insert (13). Southern blot hybridization was performed as previously reported (14). Autoradiograms were developed with Kodak X-Omat Blue film and intensifying screens for 24 h at –70°C. Films were scanned with a Umax PowerLook III scanner, and images imported and cropped in Adobe Photoshop 5.5 on a G4 Power Macintosh.

Statistical Analysis

Statistical differences at the $p < 0.05$ level among different groups were evaluated by analysis of variance with Bonferroni correction.

RESULTS

The animals were divided into three groups depending on whether the rat was treated with weekly intravenous injections of (a) saline, (b) the TH expression plasmid encapsulated in mouse IgG2a targeted PILs, or (c) the TH expression plasmid encapsulated in the OX26 TfRMAb-targeted PILs. The body weights of the animals in the three treatment groups are shown in Fig. 1, and there was no significant difference between the body weights of the animals in the three groups throughout the treatment period.

The results of the chemistry analysis of the serum taken 3 days after the sixth weekly injection are shown in Table I. There are no significant differences in any of the 14 different serum chemistries for any of the three treatment groups

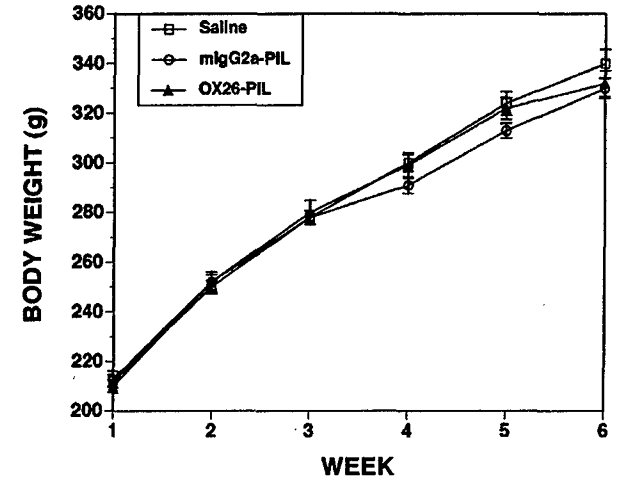

Fig. 1. The body weight of each rat in the three treatment groups was measured weekly during the course of treatment, and the mean ± SE (n = 6 rats per group) is shown. The OX26-PIL is the TfRMAb-targeted PIL, and the mIgG2a-PIL is the PIL targeted with the nonspecific mouse IgG2a, which is the isotype control antibody for the OX26 MAb.

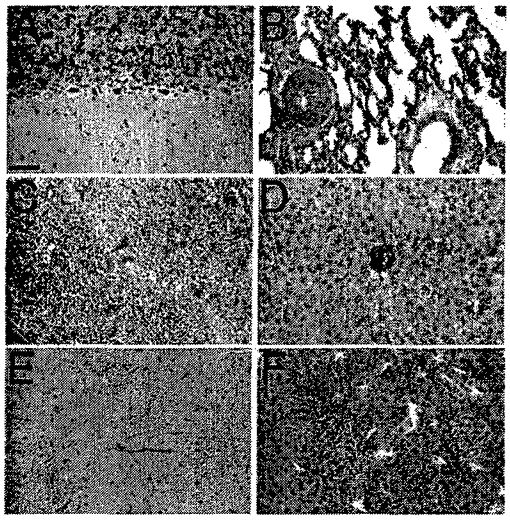

Fig. 2. Hematoxylin and eosin staining of formalin fixed cerebellum (A), lung (B), spleen (C), liver (D), heart (E), and kidney (F), removed 3 days after the sixth weekly intravenous injection of the TH expression plasmid encapsulated in the OX26 TfRMAb-targeted PIL. The magnification is the same in all panels, and the *magnification bar* in panel A is 37 μm.

(Table I). The organ histology in the rats sacrificed 3 days following the sixth weekly treatment is shown in Fig. 2 for brain cerebellum (Fig. 2A), lung (Fig. 2B), spleen (Fig. 2C), liver (Fig. 2D), heart (Fig. 2E), and kidney (Fig. 2F). The histology shown in Fig. 2 is for organs removed from rats treated with the TfRMAb-PIL. The organ histology of these animals was normal (Fig. 2) and was no different from the histology of organs taken from animals treated with either saline or the mIgG2a-PIL.

The results of the brain immunocytochemistry are given in Table II. No OX1-immunoreactive leukocytes were found in brain in any of the three treatment groups, although there was immunopositive choroidal endothelium staining in all groups (Table II). There was an occasional OX6-immunoreactive class II antigen-presenting cell in the meningial surface of all three treatment groups with no evidence of any parenchymal infiltration of class II immunopositive cells in any of the treatment groups (Table II). OX18 immunoreactivity indicative of the class I MHC antigen was found on capillary endothelium and in focal subependymal microglia, and the same staining pattern was found in all three treatment groups (Table II). OX35-immunoreactive CD4 lymphocytes were rare in brain with the same pattern in all three treatment groups (Table II). OX42-immunoreactive microglia were found diffusely in the parenchyma throughout the cerebrum and cerebellum, with an identical pattern in all treatment groups (Table II). Immunoreactive GFAP astrocytes were found diffusely throughout the cerebrum and cerebellum, with the same pattern in all three treatment groups (Table II). There was no immunoreactivity in brain with the nonspecific mouse IgG1 (mIgG1), which is the isotype control antibody for the OX1, OX18, OX6, and the GFAP antibodies (Table II). There was no immunocytochemical staining of brain using the nonspecific mouse IgG2a (mIgG2a), which is the isotype control antibody for the OX35 and OX42 antibodies (Table II).

Table I. Summary of Serum Chemistry

Assay	Units	Saline	mIgG2a-PIL	OX26-PIL
Sodium	mM	143 ± 1	142 ± 1	140 ± 1
Potassium	mM	4.4 ± 0.1	4.6 ± 0.1	4.6 ± 0.2
Chloride	mM	100 ± 1	100 ± 1	100 ± 1
CO_2	mM	29 ± 1	29 ± 1	27 ± 1
Glucose	mg/dl	168 ± 8	160 ± 6	163 ± 4
Creatinine	mg/dl	0.45 ± 0.03	0.40 ± 0.01	0.45 ± 0.02
Urea nitrogen	mg/dl	19 ± 1	21 ± 2	18 ± 1
Total protein	g/dl	5.2 ± 0.1	5.3 ± 0.1	5.3 ± 0.1
Albumin	g/dl	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Bilirubin, total	mg/dl	0.35 ± 0.03	0.25 ± 0.05	0.33 ± 0.02
Alk phos	U/L	231 ± 27	212 ± 25	281 ± 11
AST (SGOT)	U/L	65 ± 5	59 ± 2	74 ± 6
ALT (SGPT)	U/L	54 ± 2	52 ± 3	59 ± 1
Calcium	mg/dl	9.4 ± 0.1	9.5 ± 0.2	9.2 ± 0.1

Data are mean ± SE (n = 6 rats in each of the three treatment groups).

The delivery of the TH expression plasmid to brain was verified with Southern blotting as shown in Fig. 3 (Lane 3). No signal was detected in the saline-treated animals (Lane 1, Fig. 3) because these animals were not administered DNA. No hybridization signal was detected in the brain of animals treated with the expression plasmid encapsulated in the mIgG2a-PIL (Lane 2, Fig. 3) because this isotype control antibody was unable to target the PIL across the BBB and into brain cells.

DISCUSSION

These studies show that the repeat weekly intravenous administration of the PIL-based gene therapy in rats for 6 weeks causes no measurable toxicity in brain or peripheral tissues. In addition, these studies show that the chronic weekly intravenous administration of a TH expression plasmid encapsulated in TfRMAb-PILs causes no inflammation within the target organ, the central nervous system (CNS).

Table II. Summary of Immunocytochemistry

Antibody	Parameter	Fixative	Findings
OX1	Leukocytes	Methanol	Positive choroidal endothelium Same pattern in all 3 treatment groups
OX6	Class II MHC	Para.[a]	Occasional positive cell in meninges Same pattern in all 3 treatment groups
OX18	Class I MHC	Methanol	Weak staining of capillary endothelium Focal subependymal microglia Same pattern in all 3 treatment groups
OX35	CD4-lymphocytes	Methanol	Minimal staining of brain and equal to mouse IgG2a control Same pattern in all 3 treatment groups
OX42	Macrophages	Para.	Diffuse immunoreactive microglia throughout cerebrum and cerebellum Same pattern in all 3 treatment groups
GFAP	Astrocytes	Para.	Diffuse immunoreactive astrocytes throughout cerebrum and cerebellum Same pattern in all 3 treatment groups
Mouse IgG1	Control	Methanol	No reaction (control for OX1, OX18)
		Para.	No reaction (control for OX6, GFAP)
Mouse IgG2a	Control	Methanol,	No reaction (control for OX35)
		Para.	No reaction (control for OX42)

Para., paraformaldehyde.

There is no general systemic toxicity following weekly PIL administration based on the observation that the body weights of the animals increase over the 6 week treatment period at the same rate for all 3 treatment groups (Fig. 1). The PIL targets the plasmid DNA to TfR-rich organs such as the brain, liver, or spleen (9,14). The serum chemistries show normal hepatic function tests and an absence of an increase in serum bilirubin or liver enzymes (Table I). In contrast, the intravenous injection of adenovirus in primates results in increased liver enzymes secondary to hepatic inflammation caused by reaction to the immunogenic viral vector (18). There is no change in serum electrolytes or other renal function tests (Table I). The normal serum chemistry is paralleled by the normal organ histology for liver, speen, kidney, heart, lung, and brain (Fig. 2). The serum chemistry and organ histology were examined at 3 days following the sixth weekly injection because prior work has shown the TH gene expression following PIL injection is maximal at this time (13).

The intracerebral injection of viral vectors, such as adenovirus or Herpes simplex virus, leads to inflammation of the brain, as evidenced by perivascular cuffing with lymphocytes and increased immunoreactivity for class I and class I MHC antigens in brain (2–5). Therefore, the present studies performed a detailed immunocytochemical analysis of brain to examine for any evidence of inflammation in the brain following the chronic delivery to brain of a TH expression plasmid encapsulated in a TfRMAb-targeted PIL. The brain immunocytochemistry of the animals treated weekly with the TfRMAb-targeted PIL was compared to that of control groups of rats treated weekly with either saline or with mIgG2a-targeted PILs. There is an identical pattern of immunoreactivity in rat brain using OX1, OX6, OX18, OX35, OX42, and GFAP antibodies in immunocytochemical analysis of brain for all three treatment groups (Table II). In these studies, the brain was fixed with either methanol or paraformaldehyde, depending on which was the optimal fixative for each antigen (Methods), to preserve antigen recognition in the fixed brain. Chronic delivery of TfRMAb-targeted PILs to brain caused (a) no elevations in parenchymal class I (OX18) or II MHC (OX6), (b) no elevations in parenchymal infiltration by lymphocytes (OX35), leukocytes (OX1), or macrophages (OX42), and (c) no elevations in parenchymal gliosis (GFAP).

In summary, these studies demonstrate that nonviral expression plasmids can be delivered to organs with the PIL gene transfer method without toxic side effects when administered at a PIL-encapsulated plasmid DNA dose of 25 μg/kg. The chronic weekly intravenous administration of this dose of plasmid DNA encoding for rat TH and encapsulated in TfRMAb-targeted PILs causes no evidence of toxicity in ei-

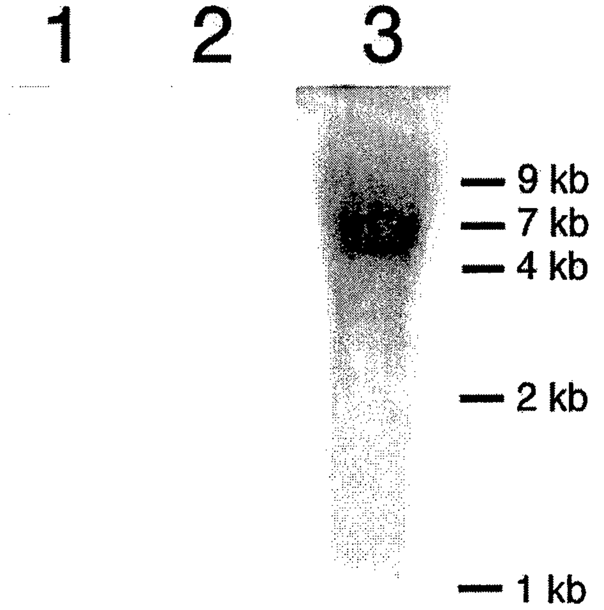

Fig. 3. Southern blot analysis of rat brain with [^{32}P]pGL2 clone 734. Lane 1, brain isolated from a saline-treated rat; lane 2, brain isolated from a rat injected with the TH expression plasmid encapsulated in the mIgG2a-targeted PIL; and lane 3, brain isolated from a rat injected with the TH expression plasmid encapsulated in the TfRMAb-targeted PIL. The migration of the DNA standards is indicated in the figure. The expected ~7-kb plasmid DNA corresponding to the size of the TH expression plasmid is seen only in the brain of the rats treated with the TfRMab-targeted PIL (lane 3).

ther the target organ, brain, or in peripheral tissues, such as liver, spleen, kidney, heart, or lung. It is possible that toxic effects may be observed at higher doses, but the dose used in this study in rats was chosen because this dose is therapeutic in rats (13). Moreover, a much higher dose, 200 μg/kg, of PIL-encapsulated plasmid DNA has been administered weekly to mice without evidence of toxicity (12). The need for high dosing of plasmid DNA with the PIL gene-targeting method is unlikely because a dose of 12 μg/kg of PIL-encapsulated plasmid DNA in adult primates results in levels of gene expression that are 50-fold higher than in rodents (10). The finding of a lack of toxicity following chronic PIL administration is important because the PIL gene transfer method delivers to the target organ a nonviral plasmid that directs gene expression for only a finite duration (12,13). The expression plasmid is transcribed episomally and is not permanently or randomly integrated into the host genome. Therefore, in order to sustain a pharmacologic effect with plasmid DNA-based gene therapy, it is necessary to administer the gene medicine on a chronic basis. The frequency of the administration is a function of the persistence of plasmid expression in the target organ. Long-term gene expression is possible with viral vectors that permanently integrate into the host genome, but this approach is associated with the risk of insertional mutagenesis (1). An alternative approach to gene therapy is chronic treatment with episomal-based plasmid DNA that is formulated in such a way that the DNA is able to target distant sites following intravenous administration. Prior work has shown that the PIL gene-targeting method enables widespread expression of the exogenous gene in distant sites such as brain in mice, rats, and rhesus monkeys (9–11). The present studies show that PIL-based gene therapy can be given chronically without the development of tissue toxicity in either the target organ, brain, or in peripheral tissues.

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Appendix 2

Normalization of Striatal Tyrosine Hydroxylase and Reversal of Motor Impairment in Experimental Parkinsonism with Intravenous Nonviral Gene Therapy and a Brain-Specific Promoter

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ABSTRACT

The goal of this work was to normalize striatal tyrosine hydroxylase (TH) activity with intravenous nonviral TH gene therapy and at the same time eliminate ectopic TH gene expression in peripheral organs such as liver in the rat. TH-expression plasmids, containing either the SV40 promoter or the glial fibrillary acidic protein (GFAP) gene promoter, were globally delivered to the brain across the blood-brain barrier (BBB) after intravenous administration of pegylated immunoliposomes (PILs). The GFAP-TH– or SV40-TH–expression plasmids were encapsulated in the interior of 85-nm PILs, which were targeted across both the BBB and the neuronal cell membrane with a monoclonal antibody (mAb) to the transferrin receptor (TfR). Striatal TH activity was 98% depleted with the unilateral intracerebral injection of 6-hydroxydopamine. TH in the striatum ipsilateral to the lesion was normalized 3 days after the intravenous injection of 10 μg per rat of either the SV40-TH or the GFAP-TH plasmid DNA. Whereas the SV40-TH gene caused a 10-fold increase in hepatic TH activity, there was no increase in liver TH with the GFAP-TH gene. The GFAP-TH gene therapy caused an 82% reduction in apomorphine-induced rotation in the lesioned rats. Confocal microscopy using antibodies to TH, GFAP, and neuronal nuclei (NeuN) showed the GFAP-TH gene was selectively expressed in nigra-striatal neurons, with no expression in either cortical neurons, or astrocytes. These studies demonstrate that global delivery of exogenous genes to the brain is possible with intravenous nonviral gene transfer, and that ectopic gene expression is eliminated with the use of brain-specific gene promoters.

OVERVIEW SUMMARY

Nonviral gene transfer using pegylated immunoliposomes (PILs) targets the plasmid DNA to brain with receptor-specific transport ligands, which act as a molecular Trojan horse to ferry the gene across both the blood-brain barrier and the neuronal cell membrane. Because the transport ligands may also deliver the gene to nonbrain organs, brain-specific gene expression can be achieved with the combined use of the PIL gene delivery technology and organ-specific gene promoters. The present work shows the ectopic expression of an exogenous tyrosine hydroxylase (TH) gene in peripheral tissues such as liver is eliminated, when the TH-expression plasmid is driven by a brain-specific promoter taken from the 5′-flanking sequence of the glial fibrillary acidic protein (GFAP) gene. After intravenous administration of PILs carrying the GFAP-TH–expression plasmid, striatal TH activity is completely restored in the 6-hydroxydopamine model of experimental Parkinson's disease.

INTRODUCTION

PARKINSON'S DISEASE (PD) is associated with a loss of dopaminergic neurons originating in the substantia nigra and terminating in the striatum (Mouradian and Chase, 1997; Mandel *et al.*, 1999). The rate-limiting enzyme in dopamine synthesis is tyrosine hydroxylase (TH), and one approach to the

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treatment of PD is TH replacement gene therapy. The goals of TH gene therapy in PD are (1) the delivery of the TH gene to the majority of the nigral-striatal neurons resulting in the restoration of dopaminergic neurotransmitter release in the striatum and (2) the selective expression of the TH gene in this region of brain without ectopic TH gene expression in either the cortex or in nonbrain organs. Ectopic TH gene expression could lead to unwanted increased dopaminergic activity in peripheral organs. The transduction of the majority of the nigral-striatal neurons with TH gene therapy is possible with a transvascular route to the brain. In this approach, the gene is administered intravenously followed by entry into the brain across the blood-brain barrier (BBB). Because every neuron is perfused by its own blood vessel, the gene is targeted to virtually every neuron in the brain following a transvascular delivery route (Pardridge, 2002). Prior work with a nonviral expression plasmid driven by the widely expressed SV40 promoter demonstrated normalization of striatal TH activity in the 6-hydroxydopamine (6-OHDA)–lesioned rat brain using the pegylated immunoliposome (PIL) gene targeting technology (Zhang *et al.*, 2003a). The TH expression plasmid is encapsulated in an 85-nm pegylated liposome, which is targeted across both the BBB and the neuronal cell membrane with a peptidomimetic monoclonal antibody (mAb) to the transferrin receptor (TfR) after intravenous administration. The transduction of rat brain with the TH-PIL gene therapy was confined to the nigral-striatal tract, and TH was not increased in the cortex of rat brain (Zhang *et al.*, 2003a). The lack of TH gene expression in the cortex is caused by the obligatory requirement of the TH enzyme for the biopterin cofactor (Hwang *et al.*, 1998). The rate-limiting enzyme in the biosynthetic pathway of biopterin, GTP cyclohydrolase I (GTPCH), is not expressed in cortex because the expression of this gene in brain is confined to monoaminergic neurons (Shimoji *et al.*, 1999). However, GTPCH is expressed in peripheral tissues such as liver (Nagatsu *et al.*, 1997). Consequently, intravenous TH gene therapy with the PIL technology and a widely expressed SV40 promoter led to ectopic gene expression in rat liver (Zhang *et al.*, 2003a). Ectopic expression of an exogenous TH gene under the influence of a widely expressed promoter is expected in any tissue that also expresses the GTPCH gene.

Ectopic TH gene expression can be reduced with the combined use of the PIL gene targeting technology and brain-specific promoters. The peripheral expression of a β-galactosidase expression plasmid was eliminated when the transgene was driven by the 2 kb of the 5′-flanking sequence (FS) of the human glial fibrillary acidic protein (GFAP) gene (Shi *et al.*, 2001a). Genes under the influence of the GFAP promoter are selectively expressed in brain, although the GFAP promoter enables gene expression in both neurons and astrocytes in brain (Kaneko and Sueoka, 1993; Galou *et al.*, 1994). Because astrocytes do not express GTPCH or the biopterin cofactor (Nagatsu *et al.*, 1997; Hwang *et al.*, 1998), it is possible the use of the GFAP promoter, in lieu of the SV40 promoter, may enable neuronal expression of the TH gene in brain, yet eliminate ectopic TH gene expression in nonbrain organs such as liver. Therefore, the purpose of the present studies was to produce a TH-expression plasmid under the influence of the GFAP promoter, and to treat 6-OHDA–lesioned rats with intravenous administration of the GFAP-TH plasmid DNA encapsulated in antitransferrin receptor monoclonal antibody (TfRmAb)-targeted PILs.

MATERIALS AND METHODS

Materials

POPC (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine) and DDAB (dimethyldioctadecylammoniumbromide) were purchased from Avanti-Polar Lipids Inc. (Alabaster, AL). Distearoylphosphatidylethanolamine (DSPE)-PEG^{2000} was obtained from Shearwater Polymers (Huntsville, AL), where PEG^{2000} is 2000-Da polyethyleneglycol. DSPE-PEG^{2000}-maleimide (MAL) was custom synthesized by Shearwater Polymers. [α-^{32}P]dCTP (3000 Ci/mmol) and [3,5-^{3}H]-L-tyrosine (51.5 Ci/mmol) were from NEN Life Science Product Inc. (Boston, MA). N-succinimidyl[2,3-^{3}H]propionate (^{3}H-NSP, 101 Ci/mmol) and protein G Sepharose CL-4B were purchased from Amersham-Pharmacia Biotech (Arlington Heights, IL). The nick translation system was from Life Technologies Inc. (Rockville, MA). The 6-OHDA, apomorphine, pargyline, catalase, (6R)-5,6,7,8-tetrahydrobiopterin (BH4), β-NADPH, L-tyrosine and charcoal were purchased from Sigma (St. Louis, MO); 2-iminothiolane (Traut's reagent) and bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce Chemical Co. (Rockford, IL). Mouse myeloma ascites containing mouse IgG2a (mIgG2a) isotype control was from the Cappel Division of ICN Pharmaceuticals (Aurora, OH). The TfRmAb used in this study is the murine OX26 mAb to the rat TfR, which is a mouse IgG2a. The anti-insulin receptor mAb used for gene targeting to human U87 glioma cells is the murine 83-14 mAb to the human insulin receptor (HIR). The TfRmAb, the HIRmAb, or the mIgG2a were individually purified with protein G affinity chromatography from hybridoma generated ascites, where HIRmAb is a murine mAb to the HIR. The pGfap-cLac plasmid (Brenner *et al.*, 1994; Segovia *et al.*, 1998) was provided by Dr. Jose Segovia, Centro de Investigacion y de Estudios Avanzados (San Pedro Zacatenco, Mexico). A mouse monoclonal antibody against GFAP (clone G-A-5), a mouse monoclonal anti-TH antibody, mouse IgG1 isotype control, and control rabbit IgG were purchased from Sigma. A mouse monoclonal antibody (MAB377) against neuronal nuclei (NeuN), a mouse mAb (MAB5262) antineurofilament 200-kd antibody, and an affinity purified rabbit polyclonal antibody (AB152) against TH were obtained from Chemicon (Temecula, CA). Secondary antibodies used were Alexa fluor 488 donkey anti-mouse IgG and Alexa fluor 594 donkey anti-rabbit IgG (Molecular Probes, Eugene, OR).

Construction of TH expression plasmid with GFAP promoter

The approximately 2-kb human GFAP promoter was obtained by polymerase chain reaction (PCR) amplification using the pGfap-cLac plasmid. Custom oliogdeoxynucleotide primers were designed to amplify nucleotides 1-2210 of the human GFAP promoter region (GenBank accession # M67446). The forward, ATGGCTAGCGAGCTCCCACCTCCCTCTCT G, and reverse, ATGAAGCTTGCGAGCAGCGGAGGTG ATGCG, primers contain *Nhe*I and *Hind*III sites, respectively, for directional cloning (Fig. 1A). In addition, these primers have three unrelated nucleotides on the 5′ end to facilitate restriction endonuclease digestion. Custom primers were obtained from Biosource International (Camarillo, CA). PCR amplification of the GFAP promoter was performed using 50 ng plasmid DNA

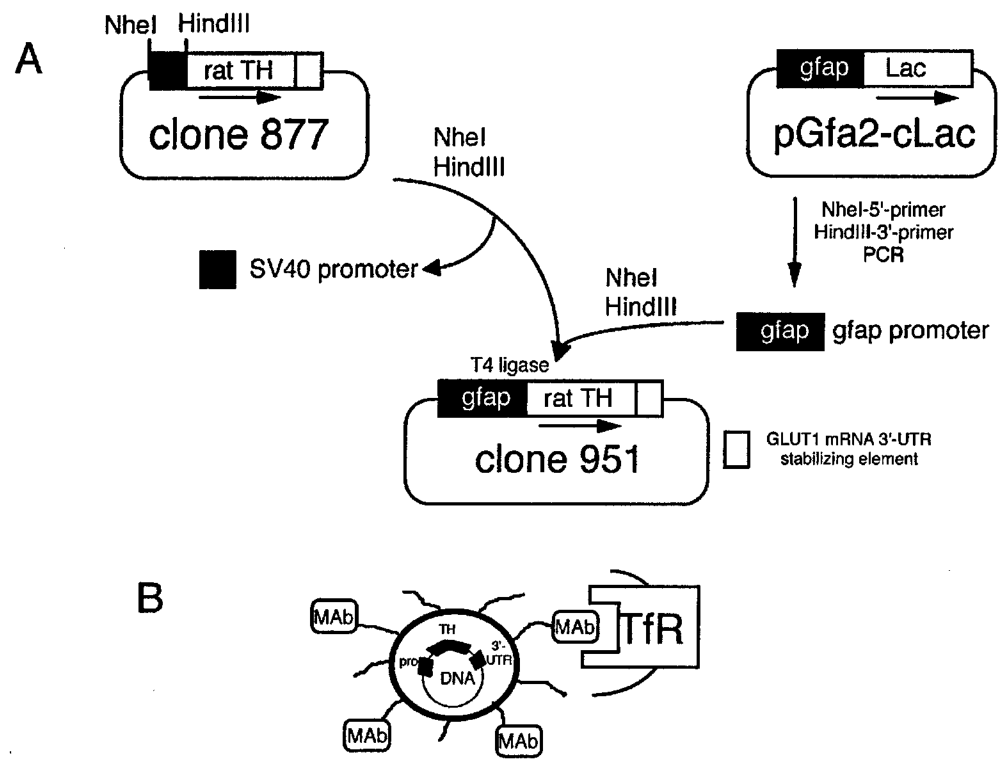

FIG. 1. A: Production of clone 951 from clone 877 and pGfa2-cLac. Clone 877 is an SV40 promoter (pro) rat tyrosine hydroxylase (TH) expression plasmid derived from pGL2 as described previously (Zhang *et al.*, 2003a). The pGfa2-cLac is a human glial fibrillary acidic protein (GFAP) promoter driven β-galactosidase expression plasmid (Brenner *et al.*, 1994; Segovia *et al.*, 1998). The rat SV promoter was released from clone 877 with *Nhe*I and *Hind*III, in parallel with the polymerase chain reaction (PCR) amplification of the GFAP promoter with an *Nhe*I 5′-primer and an *Hind*III 3′-primer. The SV40 promoter of clone 877 was replaced with the GFAP promoter to produce clone 951. Both clones 877 and 951 contain a 200 nucleotide sequence within the 3′ untranslated region (UTR), which is taken from the 3′-UTR of the bovine GLUT1 glucose transporter mRNA, and that optimizes gene expression via mRNA stabilization (Boado and Pardridge, 1998; Zhang *et al.*, 2003a). **B**: Diagram of a super-coiled TH expression plasmid encapsulated in an 85-nm pegylated immunoliposome (PIL) targeted to the rat transferrin receptor (TfR) with the OX26 murine monoclonal antibody (mAb).

in a total volume of 50 μl Pfu DNA polymerase buffer (Stratagene, La Jolla, CA). The reaction also contained 0.4 μM forward and reverse primers, 0.2 mM deoxynucleotide triphosphates (dNTPs), and 1 μl is equal to 2.5 U Pfu-Turbo DNA polymerase (Stratagene). The sample was denatured for 30 sec at 95°C, and amplified in 17 cycles of 30 sec at 95°C, 60 sec at 55°C (annealing), and 6 min at 68°C (extension). PCR products were resolved by agarose gel electrophoresis and a major band of approximately 2 kb corresponding to the human GFAP promoter was seen. PCR products were purified using the Qiagen PCR purification kit (Valencia, CA), and double-digested with *Nhe*I and *Hind*III for subcloning in the TH expression vector as described below.

The expression plasmid containing the complete open reading frame of the rat TH driven by the SV40 promoter is derived from the pGL2 plasmid and is designated clone 877 as described previously (Zhang *et al.*, 2003a). The SV40 promoter was deleted from clone 877 by double-digestion with *Nhe*I and *Hind*III, which cleaved at sites located upstream and downstream of the promoter, respectively (Fig. 1A). The approximately 6.0-kb rTH-vector backbone fragment was purified by gel electrophoresis followed by centrifugation with a Spin X filter unit. The approximately 2-kb GFAP promoter was prepared as described above and subcloned at the same restriction endonuclease sites to form a GFAP-TH–expression plasmid named clone 951 (Fig. 1A). Positive clones were identified by restriction endonuclease mapping (i.e., *Nhe*I and *Hind*III), and its identity confirmed by DNA sequencing using the pGL2 sequencing primer 1 (Promega, Madison, WI).

Synthesis of PILs

The GFAP-TH (clone 951) plasmid DNA or the SV40-TH (clone 877) plasmid DNA was encapsulated in PILs as described previously (Shi *et al.*, 2001a; Zhang *et al.*, 2002a, 2003a). The liposome is 85–100 nm in diameter and the surface of the liposome is conjugated with several thousand strands of 2000-Da polyethyleneglycd (PEG). The tips of approximately 1–2% of the PEG strands are conjugated with either the mouse OX26 mAb to the rat TfR or the mouse 83-14 mAb to the HIR or mIgG2a (Fig. 1B). The mIgG2a is the isotype control for either the murine OX26 or 83-14 mAb. Any plasmid DNA not encapsulated in the interior of the liposome is quantitatively removed by exhaustive nuclease treatment (Shi *et al.*, 2000). In a typical synthesis, 36–40% of the initial plasmid DNA (250 μg) was encapsulated within 20 μmol of lipid, and each liposome had a range of 69–73 mAb molecules conjugated to the PEG strands. The PIL conjugated with the OX26 TfRmAb is designated TfRmAb-PIL; the PIL conjugated with the murine 83-14 HIRmAb is designated the HIRmAb-PIL; and the PIL conjugated with the mouse IgG2a isotype control antibody is designated mIgG2a-PIL. The targeting mAbs are species specific and the HIRmAb-PIL is used for gene delivery to human cells and the TfRmAb-PIL is used for gene targeting to rat cells (Zhang *et al.*, 2003b).

TH gene expression in cultured U87 human glioma cells

Human U87 glioma cells were plated on 60-mm collagen-treated dishes with MEM containing 10% fetal bovine serum (FBS). When the cells reached 60% confluence, the medium was removed by aspiration, and 6 ml of fresh medium containing 10% FBS was added to the cells, followed by the addition of 167 μl of the HIRmAb-PIL carrying the 951 DNA (4 μg of plasmid DNA per dish). The cells were incubated for 2, 4, or 6 days, with three dishes at each time point, for measurement of TH enzyme activity. The cells were washed three times with cold wash buffer (5 m*M* potassium phosphate buffer), and then 400 μl of sonication buffer (wash buffer with 0.2 % Triton X-100) was added to each dish. The cells were collected, and after a short vortex, the cells were sonicated for 30 sec with a Branson Sonifier Cell Disruptor Model 185 (Branson Ultrasonics Corp., Danbury, CT). The cell homogenate was centrifuged at 10,000*g* for 10 min at 4°C. TH activity was measured with 200 μl of the supernatant as described previously (Zhang *et al.*, 2003a).

6-OHDA model

Adult male Sprague-Dawley rats (supplied by Harlan Breeders, Indianapolis, IN) weighing 200–230 g were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Animals received unilateral 6-OHDA injections into the right medial forebrain bundle as described previously (Zhang *et al.*, 2003a). Each animal received pargyline 30–60 min prior to surgery (50 mg/kg in normal saline intraperitoneally). After pargyline administration, 4 μl of 2 μg/μl of 6-OHDA (freshly prepared in 0.2 μg/μl ascorbic acid) was injected over a 4-min period using a 10-μl Hamilton syringe with the following stereotaxic coordinates: 4.8 mm posterior to bregma, 1.1 mm lateral to bregma, and 8.0 mm below the dura. The syringe needle was left in place for 2 min after the injection to allow for diffusion of the toxin. Three weeks after the lesion, rats were tested for apomorphine-induced contralateral turning using 0.5 mg/kg of apomorphine injected intraperitoneally. Full (360°), contralateral rotations only were counted over 20 min starting 5 min after apomorphine administration. Those rats turning more than 160 times in 20 min, or more than 8 rotations per min (RPM), were designated as having been successfully lesioned, and were treated 1 week later with TH gene therapy. In one series of experiments, the lesioned apormorphine-responsive rats were divided into two groups. The control group in which rats received 10 μg per rat of clone 951 DNA encapsulated in mIgG2a-PIL or the treatment group in which rats received 10 μg per rat of clone 951 DNA encapsulated in TfRmAb-PIL. The PIL was administered via the femoral vein; 3 days later the rats were tested for apomorphine-induced rotation behavior and then sacrificed. In each group, half of the rats were used for TH immunocytochemistry, and the other half were used for confocal microscopy. In a different series of experiments, the two groups of lesioned apomorphine-responsive rats were treated with either 10 μg per rat of clone 877 DNA encapsulated in TfRmAb-PIL or 10 μg per rat of clone 951 DNA encapsulated in TfRmAb-PIL. These animals were sacrificed 3 days later for measurement of organ TH activity by a radioenzymatic assay described previously (Zhang *et al.*, 2003a).

TH assay

The TH activity assay was performed as described previously (Zhang *et al.*, 2003a), and measures the conversion of L-[3,5-^{3}H]tyrosine to both [^{3}H]-H_2O and [^{3}H]-L-DOPA in a 1:1 stoichiometric relationship; the two metabolites are separated by charcoal, which selectively binds the amino acid. For the TH assay in rat organs, liver, heart, lung, kidney, frontal cortex, and the dorsal striatum in both lesioned (ipsilateral) and nonlesioned (contralateral) sides of brain were frozen in dry ice. The counts per minute were converted to picomoles of L-DOPA on the basis of the [^{3}H]tyrosine specific activity, and the results were expressed as picomoles of L-DOPA per hour per milligram of protein.

Immunocytochemistry and confocal microscopy

The brains were removed and placed into coronal or sagittal rat brain matrices for immunocytochemistry and confocal microscopy, respectively. For immuncytochemistry, frozen sections were prepared as described previously (Zhang *et al.*, 2003a). For confocal microscopy, brain tissue was then immersion fixed in 4% paraformaldehyde in 0.01 *M* phosphate-buffered water (PBW), pH 7.4 and stored overnight at 4°C. After brief washing in 0.01 *M* PBW the brain slabs were cryoprotected in 30% sucrose in 0.1 *M* PBW, pH 7.4 for additional 12 hr at 4°C. After brief washing in 0.01 *M* PBW, the brains were placed in cryomolds filled with OCT embedding compound and rapidly frozen in powdered dry ice.

TH immunocytochemistry was performed on coronal sections by the avidin-biotin complex (ABC) immunoperoxidase method (Vector Laboratories, Burlingame, CA). Frozen sections (20 μm) were incubated in either mouse anti-TH mAb (1 μg/ml) or mouse IgG1 isotype control (1 μg/ml) overnight at 4°C. The sections were incubated in biotinylated horse anti-mouse IgG (35 μg/ml) for 30 min. After development in 3-amino-9-ethylcarbazole (AEC), sections were scanned with a UMAX PowerLook III flatbed scanner (UMAX Technologies, Dallas, TX) with transparency adapter, and then cover-slipped.

Coronal sections (20 μm) through the level of the substantia nigra, pars reticulata were cut from both hemispheres for confocal microscopy. After 30 min drying at room temperature (20°C) slides were washed and permeabilized using 0.01 *M* PBS, pH 7.4 with 0.1% Triton-X 100 (PBST). Blocking for 1–2 hr was performed with 10% preimmune donkey serum in 0.01 *M* PBST at 20°C. Primary antibodies and all control studies with isotype IgG were used as follows: 15 μg/ml mouse monoclonal anti-NeuN antibody (Liu *et al.*, 1998), 10 μg/ml mouse monoclonal anti-GFAP antibody (Debus *et al.*, 1983), 2.5 μg/ml mouse monoclonal anti-TH antibody, 1 μg/ml mouse monoclonal antineurofilament 200-kd antibody (Anderton *et al.*, 1982), and 0.4 μg/ml affinity purified rabbit polyclonal anti-TH antibody (Horger *et al.*, 1998). Sections were incubated overnight at 4°C with primary antibodies diluted in 3% bovine albumin in 0.01 *M* PBST. The secondary antibody, 488 donkey anti-mouse IgG (fluorescein-labeled) and 594 donkey anti-mouse IgG (rhodamine-labeled) were used at a concentration of 5 μg/ml diluted in 0.01 *M* PBST. After extensive washing in 0.01 *M* PBS all specimens were cover-slipped and slides stored at 4°C light protected.

Confocal imaging was performed employing a Zeiss LSM 5 PASCAL confocal microscope with dual argon and helium/neon lasers equipped with Zeiss LSM software for image reconstruction (LSM 5 PASCAL, version 3.2, Jena, Germany). All sections were scanned in multitrack mode to avoid overlap of the fluorescein (excitation at 488 nm) and rhodamine (excitation at 568 nm) channels. For acquisition of three-dimensional images, up to 20 serial images with a slice thickness between 1.6–3.7 μm were used. Pinhole size for each channel was maintained as small as possible as to ensure sufficient signal-to-noise ratio and highest spatial resolution (126–145 nm). Line density ranged from 0.19–0.45 μm using these settings. Detector gain and amplifier offset were optimized to reduce artificial background for each image. No amplifier gain was used. Three-dimensional image slices were scanned with a 1024 $\times$ 1024 resolution. All scanning parameters were kept constant. Image analysis was performed for each single image slice also in the three-dimensional data stacks. Three-dimensional images were reconstructed by projecting six consecutive planar views. To ensure a more objective measure of overlap, a colocalization feature was applied that color-codes only regions in which both channels overlap at a threshold intensity level. Intensity of the fluorescent signal was measured in an arbitrary scale ranging from 0 (no signal) to 250 (highest signal) with respect to the individual image intensity profile. The intensity level was defined individually for each dataset using the integrated colocalization software and an overlap coefficient of 1 (Manders *et al.*, 1993).

Real-time PCR

The abundance of the rat TH and the control 4F2hc transcripts were determined by reverse transcriptase (RT) and real-time PCR of total RNA isolated from brain and liver tissues of saline- and PIL-injected animals. Total RNA was obtained using the RNAqueous-4PCR isolation kit (Ambion, Houston, TX). Total RNA was used to synthesize cDNA by RT with oligo dT priming and the SuperScript system for RT-PCR (Invitrogen, San Diego, CA). The real-time PCR was performed in an iCycler equipped with the optical module (BioRad, Hercules, CA), per the manufacturer's instructions using the Bio-Rad iQ SYBR green supermix. PCR reaction was run using a modified three-step amplification protocol followed by a melting curve to confirm the production of a single PCR product. The threshold cycle number (Ct) was calculated for rat TH and rat 4Fhc using the iCycler BioRad software. The 4F2hc gene encodes the heavy chain (hc) of multiple amino acid transporters and is a common housekeeping gene that is expressed in both brain and liver (Boado *et al.*, 1999). PCR primers were designed using the Beacon Designer (Palo Alto, CA) software, and obtained from Biosource International (Camarillo, CA). The TH PCR primers (forward 5′-GCTGTCACGTCCCCAAGGTT-3′ and reverse 5′-CAGCCCGAGACAAGGAGGAG-3′) amplify a region of 220 nucleotides (nt) located at nt 449–668 of the rat TH cDNA (accession # NM_012470). The 4F2hc PCR primers (forward 5′-CCAAGGAGGAGCTATTGAA GGTA-3′ and reverse 5′-CGCCCGAACGATGATAACCA-3′) target nt 369–496 of the rat 4F2hc cDNA (accession # AB015433) to produce a DNA fragment of 128 nt.

Statistical analyses

Statistically significant differences in different treatment groups were determined by analysis of variance (ANOVA) with Bonferroni correction using program 7D of the BMDP Statistical Software package developed by the UCLA Biomedical Computing Series. A p value < 0.05 was considered significant.

RESULTS

GFAP-TH–expression plasmid activity in U87 human glioma cells targeted with the HIRmAb-PIL

Human U87 cells express GFAP (Mandil *et al.*, 2001) and also support TH gene expression in cell culture (Zhang *et al.*, 2003a). Therefore, the biologic activity of the GFAP-TH–expression plasmid (clone 951) was measured in cultured U87 cells at 2, 4, and 6 days after the single application at day 0 of the plasmid DNA encapsulated in HIRmAb-targeted PILs. Clone 951 is well expressed, and the level of TH enzyme activity in the cells is comparable whether the TH gene is under the influence of either the SV40 promoter or the GFAP promoter (Table 1).

TH enzyme activity in brain and peripheral organs in lesioned rats treated with either the SV40-TH– or the GFAP-TH–expression plasmid targeted with the TfRmAb-PIL

The intracerebral injection of 6-OHDA caused a 98% reduction in TH enzyme activity in the ipsilateral striatum compared to the contralateral or non-lesioned striatum (Table 2, saline treated rats). The TH enzyme activity in the cortex ipsilateral or contralateral to the lesion was no different, and was 98% reduced compared to the TH activity in the contralateral striatum (Table 2). The 6-OHDA–lesioned, apomorphine-responsive rats were treated with SV40-TH gene therapy using clone 877 plasmid DNA encapsulated in the TfRmAb-targeted PIL. At 3 days after the single intravenous administration of 10 μg per rat of the clone 877 plasmid DNA, the TH activity in the ipsilateral or lesioned striatum was normalized and was not significantly different from the TH enzyme activity in the contralateral striatum (Table 2). SV40-TH gene therapy caused no increase in TH in the cortex in brain, but did cause a 10-fold increase in TH enzyme activity in the liver (Table 2). In contrast, there was no increase in hepatic TH enzyme activity after intravenous administration of the GFAP-TH (clone 951) en-

Table 1. TH Activity in Cultured U87 Glioma Cells After Delivery of Either SV40 Promoter (Clone 877) or GFAP Promoter (Clone 951) TH Expression Plasmid Encapsulated in HIRmAb-Targeted PIL

	TH activity (pmol L-Dopa/hour/mg$_p$)	
Days of incubation	*Clone 877*	*clone 951*
2	214 ± 14	231 ± 10
4	1458 ± 99	1576 ± 33
6	177 ± 10	311 ± 22

Mean ± SE (n = 3 dishes per time point). Clone 877 data from Zhang *et al.* (2003a).

SE, standard error; TH, tyrosine hydroxylase; GFAP, glial fibrillary acidic protein; PIL, pegylated immunoliposomes.

capsulated in the TfRmAb-targeted PIL (Table 2). However, treatment with the GFAP-TH gene, similar to the SV40-TH gene, caused complete normalization of the TH enzyme activity in the ipsilateral striatum of the 6-OHDA–lesioned apomorphine-responsive rats (Table 2).

Reversal of motor impairment after GFAP-TH gene therapy

The lesioned rats that responded to apomorphine were separated into 2 groups and treated with clone 951 plasmid DNA (10 μg/rat) encapsulated in either mIgG2a-targeted PILs or TfRmAb-targeted PILs, and apomorphine-induced rotation behavior was measured in individual rats before and 3 days after intravenous gene therapy (Figure 2A,B). The apomorphine-induced rotation (mean RPM of 4 ± 3) in the rats treated with clone 951 encapsulated in the TfRmAb-PIL was reduced 82% compared to the apomorphine-induced roation (mean RPM of 22 ± 3) in the rats treated with clone 951 encapsulated in the mIgG2a-PIL (Figure 2C).

Neuronal expression of immunoreactive TH following GFAP-TH gene therapy

TH immunocytochemistry of coronal sections of rat brain is shown in Figure 3. There was complete normalization of the immunoreactive TH in the striatum of 6-OHDA–lesioned apomorphine-responsive rats at 3 days after a single intravenous injection of clone 951 plasmid DNA (10 μg per rat) encapsulated in the TfRmAb-targeted PIL (Fig. 3A, 3B, and 3C). However, there was minimal immunoreactive TH detected in the striatum of 6-OHDA–lesioned apomorphine-responsive rats at 3 days after a single intravenous injection of clone 951 plasmid DNA (10 μg per rat) encapsulated in the mIgG2a-targeted PIL (Fig. 3D, 3E, and 3F). The marked reduction in immunoreactive TH in the striatum of these rats correlates with the 98% reduction in striatal TH enzyme activity in the lesioned animals (Table 2).

The cellular origin of TH gene expression in the brains of lesioned rats treated with the clone 951 DNA encapsulated in TfRmAb-PILs or mIgG-PILs was examined by confocal microscopy (Fig. 4). The TH gene was selectively expressed in nerve terminals in the striatum on the side contralateral to the lesion with no overlap with NeuN immunoreactive neuronal cell bodies (Fig. 4A). Virtually all of the nerve terminals were negative for TH in the ipsilateral striatum of lesioned rats treated with clone 951 encapsulated in the mIgG2a-PIL (Fig. 4B). Conversely, the density of TH reactive nerve terminals in the ipsilateral striatum of lesioned rats treated with the clone 951 DNA encapsulated in the TfRmAb-targeted PIL was no different than in the contralateral, non-lesioned striatum (Fig. 4A and 4C). The nerve terminals in the contralateral striatum that were immunopositive for TH were generally immunonegative for the 200-kDa neurofilament protein (Fig. 4D), and there was preser-

Table 2. Tyrosine Hydroxylase in Brain and Peripheral Organs in the Rat Three Days After Intravenous Injection of Gene Therapy

Organs	*Saline (pmol/hr/mg$_p$)*	*TfRmAb-PIL/877 (pmol/hr/mg$_p$)*	*TfRmAb-PIL/951 (pmol/hr/mg$_p$)*
Ipsilateral striatum	128 ± 27	5177 ± 446*	5536 ± 395*
Contralateral striatum	6445 ± 523	5832 ± 391	5713 ± 577
Ipsilateral cortex	176 ± 30	132 ± 16	184 ± 38
Contralateral cortex	150 ± 36	150 ± 24	135 ± 25
Heart	29 ± 3	45 ± 8	31 ± 3
Liver	13 ± 2	130 ± 28*	18 ± 6
Lung	42 ± 13	74 ± 22	30 ± 6
Kidney	24 ± 2	35 ± 5	31 ± 8

*$p < 0.01$ difference from saline group (ANOVA with Bonferroni correction; n = 4 rats per group). Rats were lesioned with intracerebral injections of 6-hydroxydopamine; 3 weeks after toxin injection the rats were tested for apomorphine-induced rotation behavior; those rats resting positively to apomorphine were selected for gene therapy, which was administered intravenously 4 weeks after toxin administration; all animals were euthanized 3 days after gene administration.

ANOVA, analysis of variance.

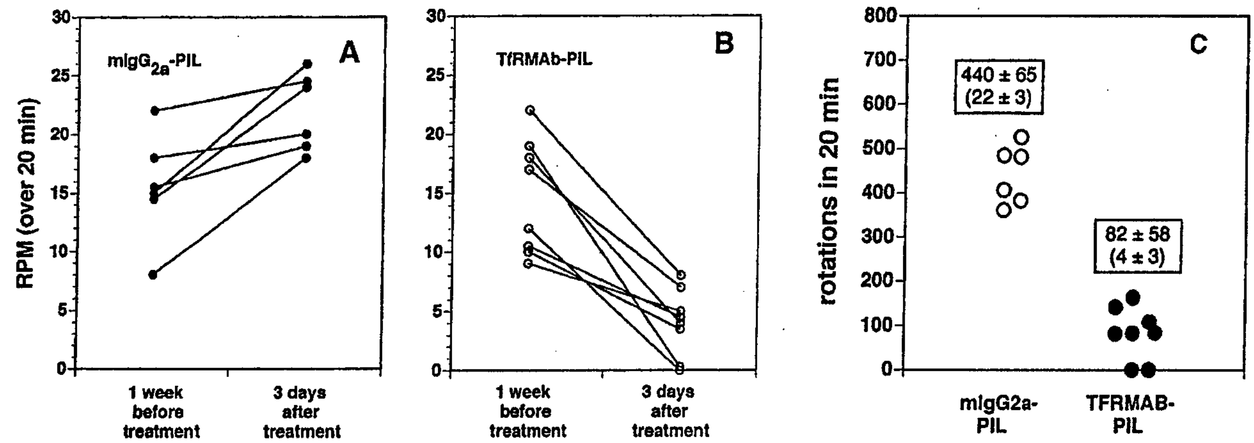

FIG. 2. **A**: Apomorphine-induced rotations per minute (RPM) over a 20-min period measured in individual rats at 1 week before treatment and at 3 days after a single intravenous injection of 10 μg per rat of clone 951 plasmid DNA encapsulated in a pegylated immunoliposome (PIL) targeted with the mouse IgG2a isotype control antibody. **B**: Apomorphine-induced RPM over a 20-min period measured in individual rats at 1 week before treatment and at 3 days after a single intravenous injection of 10 μg per rat of clone 951 plasmid DNA encapsulated in a PIL targeted with the TfRmAb. **C**: Comparison of the total rotations in the two groups at 3 days after treatment. The average RPM is 22 ± 3 and 4 ± 3 (mean ± standard deviation [SD]) in animals treated with the mIgG2a-PIL and the TfRmAb-PIL, respectively. The difference in rotation between the two groups is significant at the $p < 0.005$ level.

vation of the 200-kDa neurofilament fibers in the ipsilateral striatum of lesioned rats treated with the clone 951 encapsulated in the mIgG2a-PIL (Fig. 4E). The pattern of nerve terminals in the ipsilateral striatum of lesioned rats treated with clone 951 encapsulated in the TfRmAb-PIL was comparable to that in the contralateral striatum (Fig. 4D and 4F).

The parallel expression of immunoreactive TH, GFAP, and NeuN in the striatum and substantia nigra is shown in Figure 4 for regions of the brain ipsilateral to the lesion and in rats treated with the clone 951 plasmid DNA and encapuslated in the TfRmAb-PIL. For the striatum, separate views are shown for GFAP and TH immunostaining in Figure 4G and 4H. The overlap image in Figure 4I indicates there is no coexpression of the TH in the GFAP-positive astrocytes. Parallel immunostaining for TH and NeuN in the substantia nigra shows there is expression of immunoreactive TH in NeuN immunoreactive neuronal cell bodies (Fig. 4J, 4K, and 4L).

Measurement of TH gene expression by real-time PCR

The measurement of immunoreactive TH (Figs. 3 and 4) and TH enzyme activity (Tables 1 and 2) were corroborated by assays of TH mRNA using real-time PCR (Table 3). Rats were treated with either saline or 10 μg per rat of clone 877 plasmid

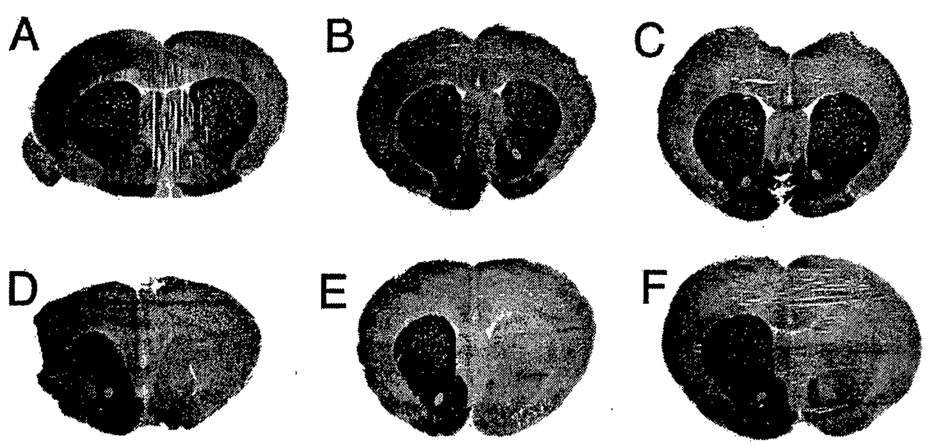

FIG. 3. Tyrosine hydroxylase immunocytochemistry of rat brain removed 72 hr after a single intravenous injection of 10 μg per rat of clone 951 plasmid DNA encapsulated in pegylated immunoliposome (PIL) targeted with either the TfRmAb (**A**, **B**, **C**) or with the mouse IgG2a isotype control (**D**, **E**, **F**). Coronal sections are shown for three different rats from each of the two treatment groups. The 6-hydroxydopamine was injected in the medial forebrain bundle of the right hemisphere, which corresponds to right side of the figure. Sections are not counterstained.

DNA encapsulated in TfRmAb-targeted PILs, and 3 days later animals were sacrificed for isolation of RNA from either cortex or liver. There was no significant increase of TH mRNA in brain cortex, although the Ct was increased from 29.0 ± 2.3 to 34.6 ± 1.3 in the livers of rats treated with TH-PIL gene therapy; this corresponds to more than a 10-fold increase in liver TH mRNA. The hepatic mRNA levels of a housekeeping gene, 4F2hc, were also measured and TH PIL gene therapy caused no change in 4F2hc mRNA concentrations in liver (Table 3).

DISCUSSION

The results of these studies are consistent with the following conclusions. First, the ectopic expression of the TH gene in peripheral tissues such as liver after intravenous gene therapy is eliminated when the TH gene is under the influence of a brain specific promoter, such as the GFAP promoter (Table 2). Second, intravenous GFAP-TH gene therapy with clone 951 (Fig. 1A) and the PIL gene targeting technology (Fig. 1B) causes a normalization of striatal TH in the 6-OHDA–lesioned rat, but this is associated with no change in cortical TH (Table 2, Fig. 3). Third, the normalization of striatal TH with intravenous GFAP-TH gene therapy is associated with an 82% reduction in apomorphine-induced rotation behavior (Fig. 2). Fourth, the GFAP-TH gene is selectively expressed in neurons, not astrocytes, of the nigral-striatal tract (Fig. 4).

The stability of the TH enzyme is linked to the availability of the biopterin cofactor, and the expression of the TH enzyme is found only in those regions of brain that express GTPCH, the rate-limiting enzyme in biopterin biosynthesis (Nagatsu *et al.*, 1995; Hwang *et al.*, 1998; Shimoji *et al.*, 1999). However, GTPCH is also expressed in some peripheral organs such as liver (Nagatsu *et al.*, 1997), which supports TH gene expression after intravenous gene therapy with an exogenous TH gene under the influence of a widely expressed promoter, such as the SV40 promoter. There is a 10-fold increase in hepatic TH activity in rats treated with clone 877, the SV40-TH, which is encapsulated in the TfRmAb-targeted PIL (Table 2). However, the ectopic expression of the TH gene in liver is eliminated with the use of a brain-specific promoter, such as the GFAP promoter (Table 2). These results parallel prior observations on β-galactosidase reporter gene expression. If the β-galactosidase gene was under the influence of the SV40 promoter, and was administered intravenously encapsulated in a TfRmAb-targeted PIL, then the gene was expressed in both brain and TfRmAb-positive peripheral organs such as liver (Shi *et al.*, 2001a,b). However, β-galactosidase gene expression in peripheral organs of the mouse was eliminated if the gene was under the influence of the GFAP promoter (Shi *et al.*, 2001a). Similarly, in the adult rhesus monkey, ocular-specific gene expression is observed after the intravenous injection of HIRmAb-targeted PILs carrying a trans-gene driven by the rhodopsin promoter (Zhang *et al.*, 2003d).

The TfRmAb-targeted PIL delivers the TH gene to hepatocytes because the hepatic microcirculation is a sinusoidal network of highly porous capillaries, which allows free access of the 85-nm PIL to the extravascular space of liver. Conversely, peripheral organs such as heart or kidney are perfused by continuous endothelial barriers that block the egress into the extravascular compartment of the circulating PIL. The endothelium of most peripheral organs do not express sufficient amounts of TfR to enable transcapillary transport of the PIL. Consequently, there is no gene expression in these organs for either TH (Table 2) or for reporter genes such as β-galactosidase or luciferase (Shi *et al.*, 2000, 2001a,b). Unlike capillaries in peripheral tissues, the capillaries of the brain express high levels of TfR (Jefferies *et al.*, 1984; Pardridge *et al.*, 1987), which can be a conduit for the delivery of genes to rodent brain (Shi *et al.*, 2001a,b). The PIL targets the TfR, which causes receptor-mediated transcytosis across the BBB followed by receptor-mediated endocytosis across the neuronal cell membrane. In addition, PILs target the nuclear membrane and the majority of intracellular DNA is confined to the nuclear compartment at 24 hr after administration (Zhang *et al.*, 2002a).

The GFAP-TH gene is expressed in neurons in the nigral-striatal tract after the intravenous administration of the clone 951 DNA encapsulated in the TfRmAb-targeted PIL, as demonstrated by confocal microscopy (Fig. 4). Conversely, neuronal TH expression is not observed in the cortex based on either measurement of TH enzyme activity (Table 2) or immuncytochemistry (Fig. 3). In addition, the confocal mi-

FIG. 4. Confocal microscopy of striatum in 6-OHDA-lesioned rats sacrificed at 3 days after intravenous injection of clone 951 plasmid DNA encapsulated in pegylated immunoliposomes (PILs) targeted either with mouse IgG2a (**B** and **E**) or with the TfRmAb (**A**, **C**, **D**, and **F**). Panels A and D are from the striatum contralateral to toxin injection, and panels B, C, E, and F are from the striatum ipsilateral to toxin injection. Panels A–C show striatum colabeled with a mouse monoclonal antibody to neuronal nuclei (NeuN) (green) and a rabbit polyclonal antibody to tyrosine hydroxylase (TH; red). Panels D–F show striatum colabeled with a mouse monoclonal antibody to the 200-kDa neurofilament protein (green) and a rabbit polyclonal antibody to TH (red). All images were taken with a 40× objective, and the magnification bar in panel A is 20 μm. All images are three-dimensional projection views of multiple planar images. The yellow color is an artifact from the three-dimensional projection as there was no overlap observed in the single planar views. Confocal microscopy of striatum (**G–I**) and substantia nigra (**J–L**) ipsilateral to the 6-OHDA lesion in rats sacrificed at 3 days after intravenous injection of clone 951 plasmid DNA encapsulated in PILs targeted with the TfRmAb. Panels G and J show immune staining (green channel) with monoclonal antibodies to glial fibrillary acidic protein (GFAP) and neuronal nuclei (NeuN), respectively. Panels H and K show immune staining (red channel) with a rabbit polyclonal antibody to TH. The overlap image of TH and GFAP in striatum is shown in panel I; the overlap image of TH and NeuN in substantia nigra is shown in panel L. Panels G–L were photographed with a 40× objective, whereas the size of panels J–L was increased with a 2× zoom. The inset of panel L is a 100× oil immersion view of colabeling of TH (red), neuronal nuclei (NeuN) (green), and the overlap (yellow) in a neuron in the substantia nigra. The magnification bars in panels G and J are 20 and 10 μm, respectively. All images are three-dimensional projection views of multiple planar images.

croscopy shows that neuronal tracts immunopositive for the 200-kDa neurofilament protein do not express TH in the striatum of the treated rat (Fig. 4F). Neuronal expression of the TH transgene in the nigro-striatal tract is also indicated by the 82% reduction in apormorphine-induced rotation behavior following intravenous administration of the GFAP-TH in the TfRmAb-PIL (Fig. 2). The ability of intravenous TH gene therapy to cause normalization of nigral-striatal TH expression in the 6-

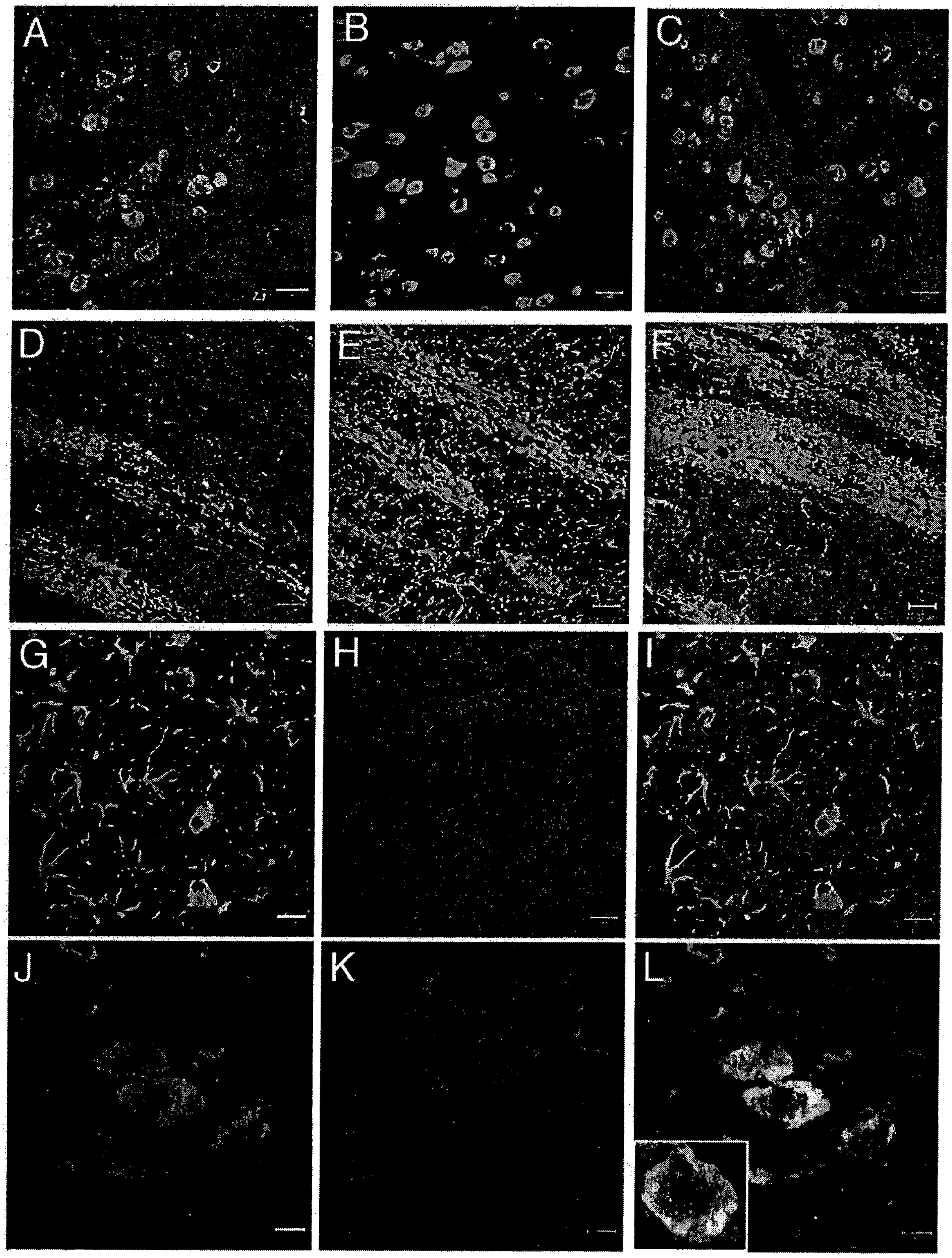

TABLE 3. REAL-TIME PCR Ct FOR TH AND 4F2hc mRNA LEVELS IN CONTROL, NONLESIONED RATS TREATED WITH EITHER SALINE OR TH GENE THERAPY

	Ct values					
	Saline			*TfRMAb-PIL/877*		
Organ	*TH*	*4F2hc*	*TH/4F2hc*	*TH*	*4F2hc*	*TH/4F2hc*
Brain cortex	26.3 ± 0.1	19.3 ± 0.2	1.36 ± 0.01	25.7 ± 0.1	19.4 ± 0.3	1.33 ± 0.02
Liver	34.6 ± 1.3	19.9 ± 0.2	1.74 ± 0.06	29.0 ± 2.3*	20.1 ± 0.3	1.43 ± 0.09*

Mean ± SE ($n = 3$ rats per group).
*$p < 0.05$ difference from saline values.
PCR, polymerase chain reaction; Ct, threshold cycle; TH, tyrosine hydroxylase.

OHDA–lesioned model may be related to the immediate regeneration in this pathway after neurotoxin administration. Providing that at least 25% of the nigral neurons survive the chemical lesion, which is the case for the moderate dose (8 μg) of 6-OHDA used in this study (see Materials and Methods), there is intense sprouting of surviving neurons from the substantia nigra to the striatum after the lesion (Finkelstein *et al.*, 2000; Parish *et al.*, 2002). Therefore, when the TH expression plasmid is delivered to substantia nigral neurons at 4 weeks after the lesion, the enzyme may be expressed in these cell bodies (Fig. 4L) and transported to the regenerated terminals in the striatum (Fig. 4C and 4F). In addition, the TH may be transported to the striatum via neurons that survive the chemical lesion.

The expression of the GFAP-TH gene in brain is confined to neurons with no expression in astrocytes (Fig. 4). The 5′-FS of the GFAP gene confers brain specificity of gene expression, but does not restrict gene expression to astrocytes (Kaneko and Sueoka, 1993; Galou *et al.*, 1994). Astrocyte-specific expression requires the coordinate interactions of regulatory elements in both the 5′-FS and more distal parts of the gene, including the 3′-FS (Kaneko and Sueoka, 1993). Recent work in transgenic models demonstrate that the 5′-FS of the GFAP gene enables widespread neuronal expression of transgenes in brain (Zhuo *et al.*, 2001). These findings parallel other observations that neurons secrete trans-acting factors that interact with the 5′-FS of the GFAP gene (Gomes *et al.*, 1999). The 5′-FS of the GFAP gene is completely methylated in peripheral tissues such as spleen but is hypomethylated in neurons and astrocytes (Condorelli *et al.*, 1997). In the presence of the entire GFAP gene, the neuron-suppressing elements in the 3′-FS prevent GFAP gene expression in neurons in brain in vivo (Kaneko and Sueoka, 1993). However, in the absence of the 3′-FS, the GFAP promoter can be used to direct brain-specific expression of exogenous genes in neurons (Zhuo *et al.*, 2001). The GFAP promoter also enables gene expression in astrocytes (Brenner *et al.*, 1994). However, no TH gene expression in astrocytes was detected in this study (Fig. 4G–4I). Similarly, the TH gene expression in cortical neurons was minimal (Fig. 3). Neither astrocytes or cortical neurons express the GTPCH gene, and do not produce the biopterin cofactor necessary for TH enzyme activity (Nagatsu *et al.*, 1995; Hwang *et al.*, 1998).

The absence of any increase in the cortex of immunoreactive TH (Fig. 3) or TH enzyme activity (Table 2) is paralleled by the absence of a change in TH mRNA in this region of the brain as measured by real-time PCR (Table 3). These results parallel the findings of the human TH transgenic mouse, wherein no increase in TH mRNA levels in the cortex were recorded (Kaneda *et al.*, 1991), and this is attributed, in part, to the absence of GTPCH gene expression in the cortex (Shimoji *et al.*, 1999). However, the expression of the exogenous TH gene in brain after delivery with PILs is expected in those neurons expressing the GTPCH cofactor gene. The use of a brain-specific promoter, such as the GFAP gene promoter, eliminates ectopic TH gene expression in peripheral tissues, but would not eliminate TH gene expression in cells of the brain that also express the GTPCH cofactor gene. The restriction of TH gene expression to only dopaminergic neurons is possible with the use of a trans-gene driven by the TH gene promoter that encompasses 9 kb of the 5′-FS of the gene (Min *et al.*, 1994).

The striatal TH enzyme activity is normalized with either the SV40 promoter (clone 877) or the GFAP promoter (clone 951), as shown in Table 2. In neither case are supranormal levels of TH enzyme activity observed. These observations in adult rats subjected to TH gene therapy parallel findings in TH transgenic mice. Despite a more than 50-fold increase in nigral TH mRNA levels, only minor increases in either immunoreactive TH or in TH enzyme activity in the striatum were observed (Kaneda *et al.*, 1991). These observations suggest that TH gene expression is controlled at the posttranscriptional level in the brain so that striatal TH enzyme activity is regulated within a narrow range (Min *et al.*, 1994).

In summary, the present study demonstrates transduction of the entire striatum with TH gene therapy in the 6-OHDA–lesioned rat brain. The global expression of the TH gene in the entire striatum is possible because the exogenous gene encapsulated in PILs is delivered to brain via the transvascular route across the BBB. With the transvascular approach to brain gene therapy, nearly every neuron in the brain is accessible to the exogenous gene after intravenous administration in either rodents (Shi *et al.*, 2001a,b) or primates (Zhang *et al.*, 2003c,d). Ectopic expression of the TH gene in peripheral organs such as liver is eliminated with the combined use of a brain-specific promoter and the PIL gene targeting technology. Brain TH gene expression is reversible and declines 50% at 6 days after a single intravenous administration (Zhang *et al.*, 2003a). The decline in gene expression with time is caused by degradation of the episomal plasmid DNA based on real-time PCR measurements (unpublished observations). Therefore, long-term TH gene therapy requires repeat administration at periods determined by the persistence in brain of plasmid gene expression. PIL gene therapy has been administered intravenously on a weekly schedule and this resulted in a 100% increase in survival time in mice with intra-cranial brain cancer (Zhang *et al.*, 2002b). The period of repeat gene administration in humans may be less frequent, as the level of gene expression in the rhesus monkey is still in the therapeutic range at 2–3 weeks after a single intravenous administration (Zhang *et al.*, 2003d). Long-term weekly administration of TH expression plasmids encapsulated in TfRmAb-targeted PILs has no toxic side effects in rats and causes no change in serum chemistry, organ histology, or body weights, and induces no inflammatory reactions in brain (Zhang *et al.*, 2003e). Southern blotting shows no temporal decline in the level of plasmid persistence in brain associated with long-term weekly intravenous treatments (Zhang *et al.*, 2003e). Long-term administration of reversible, episomal-based nonviral gene therapeutics is an alternative approach that avoids problems associated with random and permanent integration in the host genome of exogenous genes. The use of the PIL gene targeting technology enables the noninvasive delivery of the exogenous gene to all target cells within the brain after intravenous administration.

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Appendix 3

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RESEARCH ARTICLE

Organ-specific expression of the lacZ gene controlled by the opsin promoter after intravenous gene administration in adult mice

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Abstract

Background The tissue-specific expression of an exogenous gene, under the influence of a tissue-specific promoter, has been examined in the past with pro-nuclear injections of the transgene and the development of transgenic mouse models. 'Adult transgenics' is possible with the acute expression of an exogenous gene that is administered to adult animals, providing the transgene can be effectively delivered to distant sites following an intravenous administration.

Methods The organ specificity of exogenous gene expression in adult mice was examined with a bacterial β-galactosidase (LacZ) expression plasmid under the influence of the bovine rhodopsin gene promoter. The 8-kb plasmid DNA was delivered to organs following an intravenous administration with the pegylated immunoliposome (PIL) non-viral gene transfer technology. The PIL carrying the gene was targeted to organs with the rat 8D3 monoclonal antibody (MAb) to the mouse transferrin receptor (TfR).

Results The rhodopsin/β-galactosidase gene was expressed widely in both the eye and the brain of adult mice, but was not expressed in peripheral tissues, including liver, spleen, lung, or heart. Ocular expression included the retinal-pigmented epithelium, the iris, and ciliary body, and brain expression was observed in neuronal structures throughout the cerebrum and cerebellum.

Conclusions The expression of trans-genes in adult animals is possible with the PIL non-viral gene transfer method. The opsin promoter enables tissue-specific gene expression in the eye, as well as the brain of adult mice, whereas gene expression in peripheral tissues, such as liver or spleen, is not observed. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords brain; gene therapy; non-viral gene transfer; rhodopsin; liposomes; transferrin receptor

Introduction

Tissue-specific gene expression in the brain under the influence of a specific promoter is generally examined with transgenic mice following pro-nuclear injection of the gene and development of the embryo into mature animals. The expression of trans-genes in adult animals within 24 h is possible with intravenous administration and non-viral gene targeting technology. A new approach to non-viral gene transfer encapsulates plasmid DNA inside pegylated immunoliposomes (PILs) [1].

The PIL gene transfer technology has been used in adult mice, rats, and adult rhesus monkeys [2–4]. The non-viral plasmid DNA is encapsulated in the interior of an 85-nm liposome, and the surface of the liposome is conjugated with several thousand strands of 2000 Da poly(ethylene glycol) (PEG). This pegylation of the liposome stabilizes the nanocontainer *in vivo*, and allows for prolonged blood residence time [5]. The pegylated liposome is targeted across biological barriers in the brain using targeting ligands that are conjugated to the tips of 1–2% of the PEG strands. The targeting ligand may be an endogenous peptide or a peptidomimetic monoclonal antibody (MAb) that targets a cell surface receptor, and enables transport of the PIL across the membrane barrier. For gene targeting to the brain, the PIL must be delivered across both the brain capillary endothelial wall, which forms the blood-brain barrier (BBB) *in vivo*, and the brain cell plasma membrane. The transferrin receptor (TfR) is expressed at both the BBB [6] and the brain cell membrane [7]. Peptidomimetic MAbs that target the TfR enable the transport of PILs into brain cells in either rats or mice *in vivo* following intravenous administration [2,3]. Peptidomimetic MAbs that target the human insulin receptor (HIR) enable global gene expression within the adult rhesus monkey brain following an intravenous injection [4]. The exogenous gene is expressed in brain 24–48 h after gene administration.

The use of brain-specific promoters, such as the 5′-flanking sequence (FS) of the glial fibrillary acidic protein (GFAP) gene, enables brain-specific gene expression, and eliminates expression of the transgene in non-brain organs following intravenous administration [2]. In searching for other promoters that may allow for brain-specific expression of exogenous genes, the present studies examine the specificity of tissue expression of the lacZ gene that is regulated by the opsin promoter. Transgenic mice expressing the lacZ gene under the influence of the bovine opsin promoter demonstrate expression of the transgene in brain as well as structures of the eye [8]. This finding is consistent with other work showing that opsin genes are expressed in the central nervous system (CNS) [9], and that brain produces proteins that bind to the opsin promoter [10]. The present studies describe the production of PILs carrying an expression plasmid encoding the lacZ gene under the influence of the bovine opsin promoter. The PIL is injected intravenously and is targeted to brain and other organs in adult mice *in vivo* with the rat 8D3 MAb to the mouse TfR as the targeting ligand on the PIL. Gene expression in brain, eye, and peripheral tissues is measured with β-galactosidase histochemistry.

Materials and methods

Materials

Adult female BALB/c albino mice (25–30 g) were purchased from Harlan (Indianapolis, IN, USA). The β-galactosidase staining kit was purchased from Invitrogen (San Diego, CA, USA). The 8D3 hybridoma line, secreting a rat IgG to the mouse transferrin receptor, was obtained from Dr. Britta Engelhardt (Max Planck Institute, Bad Nauheim, Germany), and the 8D3 MAb was purified as described previously [2]. The 1D4 mouse monoclonal antibody against bovine rhodopsin [11] was obtained from Dr. Dean Bok (UCLA School of Medicine, Los Angeles, CA, USA). The vector mouse-on-mouse (M.O.M.) immunodetection kit, the 3-amino-9-ethylcarbazole (AEC) substrate kit for peroxidase and hematoxylin QS counter-stain were purchased from Vector Laboratories (Burlingame, CA, USA). Tissue-Tek embedding compound was purchased from Sakura FineTek (Torrance, CA, USA). All other reagents were purchased from Sigma (St Louis, MO, USA).

LacZ expression plasmid

The LacZ expression plasmid under the influence of the bovine rhodopsin promoter is designated rhodopsin/β-galactosidase, and was provided by Dr. Don Zack (Johns Hopkins University), and has been described previously [8]. This 8-kb plasmid includes nucleotides −2174 to +70 of the bovine rhodopsin gene. The presence of this portion of the bovine rhodopsin promoter within the plasmid was confirmed by DNA sequencing using a M13 reverse sequencing primer followed by custom sequencing primers. Digestion of the rhodopsin/β-galactosidase plasmid with BamHI released the 3.0-kb insert from the 5.0-kb vector backbone. In addition, the lacZ expression plasmid under the influence of the SV40 promoter, pSV-β-galactosidase (Promega), was encapsulated in 8D3 PILs, as described previously [12].

In vivo administration of PILs

The preparation of the 8D3 PIL carrying β-galactosidase expression plasmids has been described previously [2,12]. The liposome is 85–100 nm in diameter and the surface of the liposome is conjugated with several thousand strands of 2000 Da poly(ethylene glycol) (PEG). The tips of about 1–2% of the PEG strands are conjugated with the rat 8D3 MAb to the mouse TfR. Any plasmid DNA not encapsulated in the interior of the liposome is quantitatively removed by exhaustive nuclease treatment [5]. In a typical synthesis, 21% of the initial plasmid DNA (200 µg) was encapsulated within 20 µmol of lipid, and each liposome had on average 48 8D3 MAb molecules conjugated to the PEG strands.

Female BALB/c mice of 25–30 g body weight were anesthetized with ketamine (50 mg/kg) and xylazine (4 mg/kg) intraperitoneally. Experimental animals were injected intravenously through the jugular vein with 8D3 PIL carrying either the rhodopsin/β-galactosidase or the pSV-β-galactosidase plasmid DNA; the injection dose was 5 µg DNA/per mouse. In some animals, the 8D3-targeting MAb was replaced with a control rat IgG that does not recognize the TfR, as described previously [2,12]. Mice injected with the 8D3 PIL or rat IgG PIL were sacrificed at 48 h after the single intravenous injection of the gene. A total of 12 adult mice were used for this study. Organs were removed and frozen in OCT embedding medium on dry ice and stored at −70 °C.

β-Galactosidase histochemistry

β-Galactosidase histochemistry was performed on frozen sections of organs as described previously [2]. Frozen sections of 18 μm thickness were cut on an HM505 microtome cryostat (Micron Instruments, San Diego, CA, USA), and fixed with 0.5% glutaraldehyde in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 5 min. After washing in PBS, sections were incubated in X-gal staining solution (4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 1 mg/ml X-gal, pH 7.4) at 37 °C overnight, where X-gal = 5-bromo-4-chloro-3-indoyl-β-D-galactoside. The pH of the incubation was maintained at 7.4 throughout the incubation. After the staining with X-gal, sections were briefly washed in distilled water. Eye sections were lightly counter-stained with hematoxylin, whereas sections of brain, liver, spleen, heart, lung or kidney were not counter-stained. Frozen sections of mouse kidney were also processed in the histochemistry assay. Kidneys from control, uninjected mice express endogenous β-galactosidase-like enzyme activity that is active at neutral pH [3]. Therefore, including kidney sections in the β-galactosidase histochemistry assay serves as a positive internal control, and indicates the absence of histochemical product in other organs represents a lack of β-galactosidase gene expression in that organ.

Immunohistochemistry

Immunohistochemistry for rhodopsin was performed with the avidin-biotin immunoperoxidase method (Vector Labs, Burlingame, CA, USA). Frozen sections of the eyes were fixed in 2% paraformaldehyde for 20 min at 4 °C, and immunocytochemistry with the 1D4 MAb to rhodopsin was performed as described previously [12].

Results

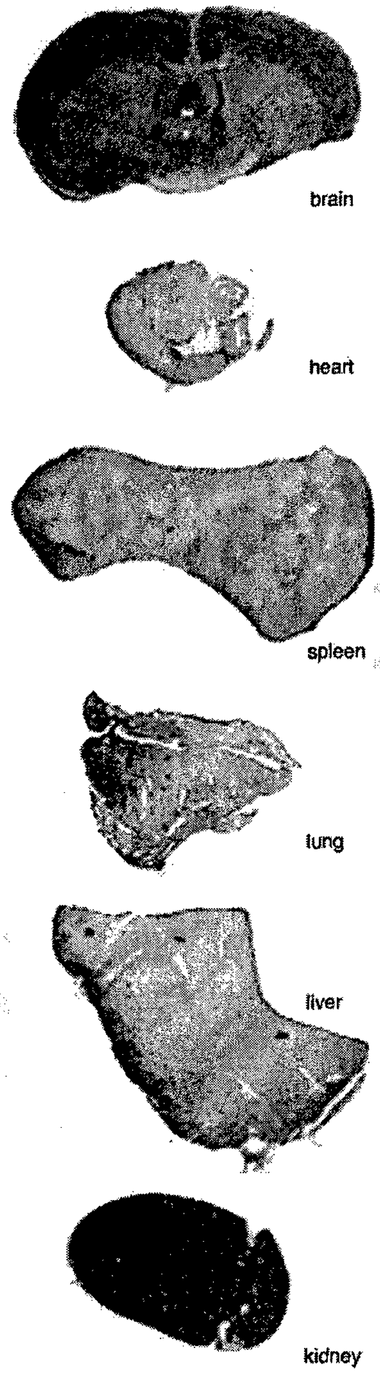
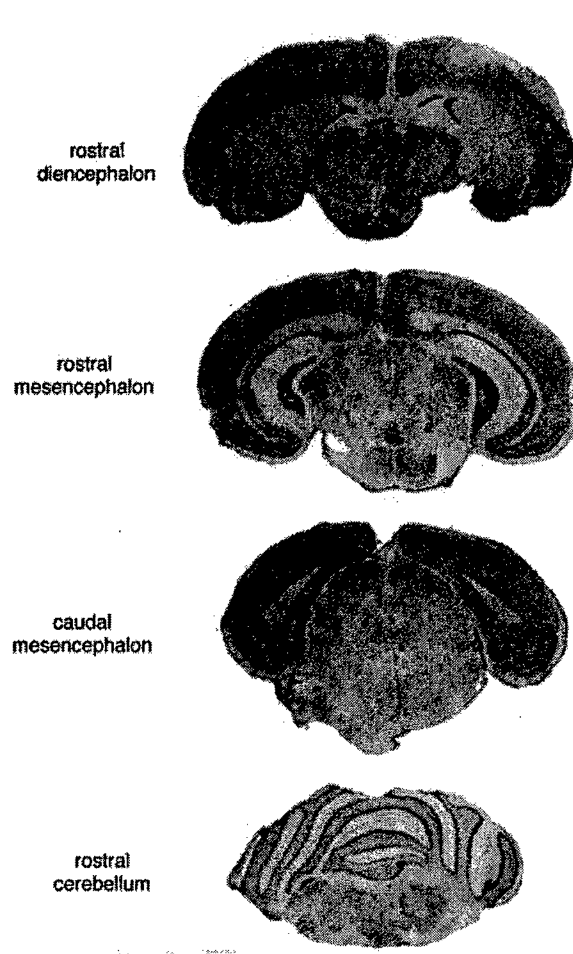


Figure 1. **β-Galactosidase histochemistry in multiple organs of adult mice sacrificed 48 h after intravenous administration of 5 μg/mouse of the rhodopsin-lacZ plasmid encapsulated in a PIL targeted with the rat 8D3 MAb to the mouse TfR. The kidney histochemistry is a positive control of the assay, as the kidney of normal mice not injected with the transgene normally expresses high amounts of β-galactosidase that is active at neutral pH**

The β-galactosidase histochemistry of brain, heart, spleen, lung, liver, and kidney is shown in Figure 1 at 48 h after the intravenous injection of 5 μg/mouse of plasmid DNA encapsulated in the PIL targeted with the rat 8D3 MAb to the mouse TfR. The lacZ gene is expressed in brain, but not in heart, spleen, lung, or liver. The β-galactosidase histochemical product in kidney does not represent exogenous gene expression, but rather demonstrates that kidney contains high amounts of endogenous β-galactosidase that is active at neutral pH [2,3]. Kidney β-galactosidase histochemistry serves as a positive control for the assay. The presence of histochemical product in kidney indicates the negative histochemical signal in heart, spleen, lung, or liver, and is not a methologic problem related to the histochemical assay, but is indicative of a lack of lacZ gene expression in these tissues.

The lacZ gene under the influence of the bovine opsin promoter is widely expressed in the mouse CNS, as shown by serial coronal sections (Figure 2). In the rostral diencephalon, lacZ gene expression is seen in the choroid plexus, the motor cortex, the thalamus/hypothalamus, and gene expression is particularly dense at the base of the brain in the region of the suprachiasmatic nucleus (SCN), which is adjacent to the third ventricle. In the rostral mesencephalon, lacZ gene expression is seen in the visual cortex, the hippocampus, the pontine gray matter, and the periaqueductal gray. In the caudal mesencephalon, lacZ expression is high in gray matter structures of the cortex, and is minimal in white matter structures of the external capsule; lacZ expression is also seen in the periaqueductal gray regions. In the rostral cerebellum, lacZ expression is high in the Purkinje cell layer and in the pons. Light microscopy of selected brain regions shows high lacZ expression in the SCN and lateral hypothalamus around the third ventricle (Figure 3A), the hippocampal dentate gyrus (Figure 3B), the hippocampal statum pyramidale (Figure 3C), the visual cortex (Figure 3D), the Sylvian aqueduct (Figure 3E), and the Purkinje cell layer of the cerebellum (Figure 3F).

The lacZ gene driven by the bovine opsin promoter is expressed in the outer retina, the iris, and the ciliary body of the eye when the PIL is targeted with the TfRMAb (Figure 4A). However, there is no lacZ gene expression in the eye when the PIL is targeted with a control isotype rat IgG (Figure 4B). Light microscopy of β-galactosidase histochemistry in the region of the outer retina is shown in Figure 4C for animals injected with the rhodopsin/β-galactosidase plasmid and in Figure 4E for animals injected with the pSV-β-galactosidase plasmid. Parallel sections were also taken for rhodopsin immunocytochemistry, as shown in Figure 4D. Rhodopsin immunoreactivity is detected in the outer segments (OS) of the retina, but not in the retinal-pigmented epithelium (RPE), the inner segments (IS), or the outer nuclear layer (Figure 4C). The parallel β-galactosidase histochemistry (Figure 4C) and the rhodopsin immunocytochemistry (Figure 4D) show that the lacZ gene is expressed only in the RPE of the mouse retina, and not in the outer nuclear layer or inner and outer segments of the retinal photoreceptor cells, following injection of 8D3 PILs carrying the rhodopsin/β-galactosidase plasmid DNA. Similarly, lacZ gene expression is confined to the RPE following injection of 8D3 PILs carrying the pSV/β-galactosidase plasmid DNA (Figure 4E).



Color Figure - Print and Online

Figure 2. β**-Galactosidase histochemistry in mouse brain 48 h after intravenous administration of 5 μg/mouse of the rhodopsin-lacZ plasmid encapsulated in a PIL targeted to brain with the 8D3 MAb to the mouse TfR. Sections through the rostral diencephalon, the rostral mesencephalon, the caudal mesencephalon, and the rostral cerebellum are shown**

Discussion

The results of these studies are consistent with the following conclusions. First, the lacZ gene driven by the bovine opsin promoter is selectively expressed in mouse brain and eye following intravenous expression, but not in peripheral tissues such as liver, spleen, heart, or lung (Figure 1). Second, structures of the brain expressing the lacZ gene under the influence of the opsin promoter include neuronal structures in the cerebral cortex (Figure 2), the hippocampus and cerebellum (Figure 3), and the epithelium lining the choroid plexus (Figure 2) or the Sylvian aqueduct (Figure 3E). Third, the lacZ gene under the influence of the opsin promoter is expressed in ocular structures of the mouse including the RPE, the iris, and ciliary body (Figure 4).

Gene expression in organs such as heart or lung is not expected following intravenous administration of TfRMAb-targeted PILs, because these organs have capillaries with continuous endothelial barriers that do not express significant levels of the TfR [3]. The circulating PIL is too large to non-specifically cross the continuous endothelial barrier, and cannot access the parenchymal cells in organs such as heart or lung

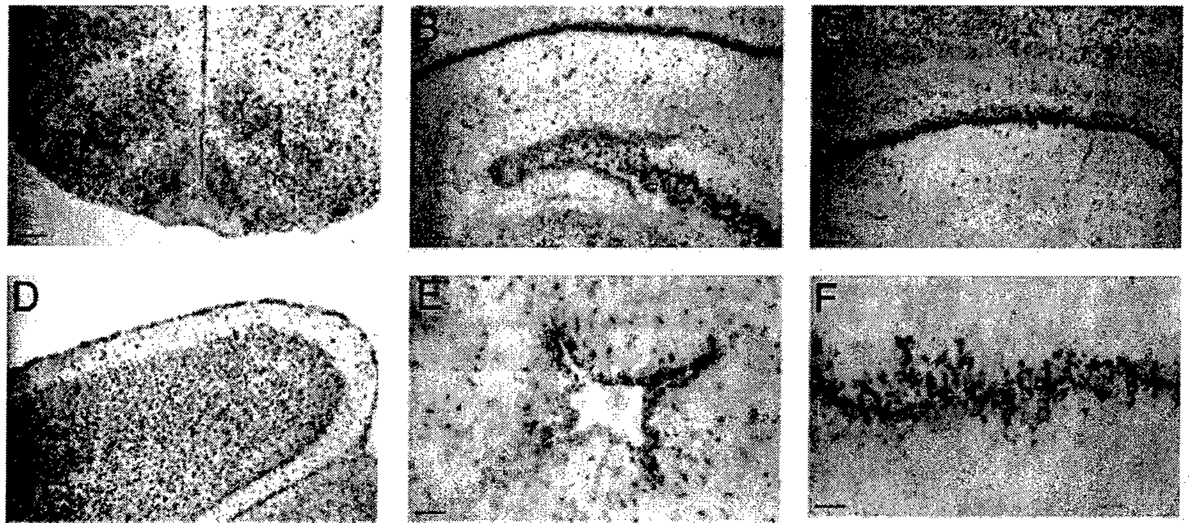


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Figure 3. Light microscopy of β-galactosidase histochemistry of mouse brain 48 h after intravenous administration of the rhodopsin-lacZ plasmid shows selective gene expression in the suprachiasmatic nucleus and lateral hypothalamus around the third ventricle (A), the dentate gyrus of the hippocampus (B), the hippocampal stratum pyramidale (C), the visual cortex (D), the epithelium lining the Sylvian aqueduct (E), and cells of the Purkinje cell layer (F). Magnification bar: 125 μm (A–D) and 28 μm (E, F)

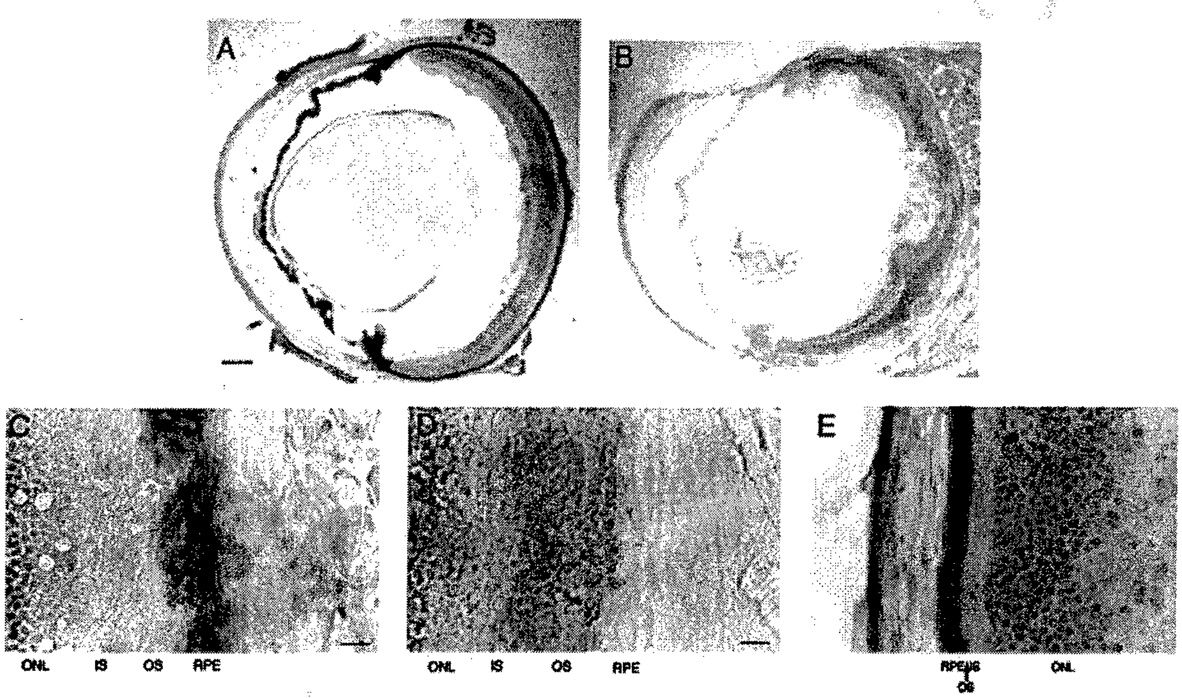

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Figure 4. β-Galactosidase histochemistry (A, B, C, E) and rhodopsin immunocytochemistry (D) in the eye of mice at 48 h after intravenous injection of 5 μg/mouse of the lacZ plasmid encapsulated in a PIL targeted with either the rat 8D3 MAb to the mouse TfR (A, C, D), or the rat IgG isotype control antibody (B); the lacZ expression plasmid was under the influence of either the bovine rhopsin promoter (A–D) or the SV40 promoter (E). (A) The principal sites of rhodopsin promoter-lacZ gene expression in the eye are the outer retina, iris, and ciliary body when the PIL is targeted to the eye with the TfRMAb. (B) When the PIL carrying the lacZ gene is targeted to the eye with a rat IgG isotype control antibody, which does not recognize any receptor, there is no lacZ histochemical product in the eye. (C, D) Comparison of the β-galactosidase histochemistry (C) and the rhodopsin immunocytochemistry (D) shows the lacZ gene is not expressed in the photoreceptor cells of the retina, which includes the outer nuclear layer (ONL), the inner segments (IS), and the outer segments (OS). The lacZ gene is expressed in the retinal-pigmented epithelium (RPE). The localization of lacZ gene expression to the RPE is comparable for either the rhodopsin promoter (C) or the SV40 promoter (E). Magnification bar: 210 μm (A) and 10 μm (C, D)

with continuous endothelium lacking endothelial TfR. In contrast, organs such as liver or spleen are perfused by sinusoidal microvasculatures that are highly porous. The PIL freely crosses the microvascular barrier in organs with sinusoidal microcirculations and enters the parenchymal space of organs such as liver or spleen. The parenchymal cells in liver or spleen express TfR, which allows for cellular uptake of the PIL. This uptake is followed by expression of the exogenous gene encapsulated within the PIL, providing the gene is under the influence of a widely expressed promoter such as the SV40 promoter [3,4]. However, the present studies show that if the lacZ gene is under the influence of an opsin promoter, there is no measurable gene expression in liver or spleen. Similar findings were reported when the lacZ gene was under the influence of the human GFAP promoter [2]. These observations suggest that the trans-acting factors that activate the opsin or GFAP promoter are not expressed in peripheral organs such as liver or spleen.

The lacZ gene under the influence of the opsin promoter is expressed in brain following the intravenous injection of the TfRMAb-targeted PIL carrying the expression plasmid (Figures 2 and 3). This observation is consistent with previous work showing that there is a family of opsin genes, and that opsin genes are expressed in the CNS. Encephalopsin is expressed in brain and particularly in the Purkinje cell layer [9]. The encephalopsin gene is homologous with the retinal opsin gene. Brain of certain species produces proteins that bind to the rhodopsin promoter [10], which is in accord with the finding of the present study that the lacZ gene under the influence of the rhodopsin promoter is expressed in mouse brain. The results of this study reproduce the findings reported previously in transgenic mice, wherein the lacZ gene under the influence of the rhodopsin promoter is expressed in transgenic mouse brain [8]. However, the lacZ gene under the influence of the bovine rhodopsin promoter is not expressed in adult rhesus monkey brain [13]. This observation suggests that the brain of higher animals may not express proteins that activate the rhodopsin promoter. The present studies in adult mice show that an exogenous gene under the influence of the bovine rhodopsin promoter, and administered intravenously, is widely expressed throughout the CNS. There is higher gene expression in gray matter as compared with white matter, and the gene is expressed in structures of both the neocortex and the paleocortex (Figures 2 and 3).

In the eye, the expression of the lacZ gene under the influence of the bovine opsin promoter is primarily expressed in the RPE, the iris, and the ciliary body, and to a lesser extent in the iridocorneal angle, the corneal endothelium, the inner retina, the lens capsule, the conjunctiva, as well as scattered cells in the ganglion cell layer of the inner retina (Figure 4A). Comparison of the β-galactosidase histochemistry with rhodopsin immunocytochemistry (Figure 4C-D) indicates the lacZ transgene is not expressed in the photoreceptor cells of the mouse eye following delivery with TfRMAb-targeted PILs. The failure to detect lacZ gene expression in the photoreceptor cells is due to the minimal TfR expression in the outer nuclear layer (ONL) [12]. The minimal expression of the TfR in the ONL of the retina parallels the very low level of iron and ferritin within this part of the retina [14]. Similarly, when the lacZ gene was targeted to adult mouse retina under the influence of the widely expressed SV40 promoter, no gene expression in the photoreceptor cells was observed [12], and this finding is replicated in the present study (Figure 4E). In contrast, high expression of the lacZ gene under the influence of either the SV40 promoter or the rhodopsin promoter is observed in the photoreceptor cells of the adult rhesus monkey 48 h after the intravenous injection of the plasmid encapsulated in PILs targeted with an MAb to the HIR [13]. The exogenous gene was expressed in the cell bodies of the ONL, and in the outer and inner segments of the photoreceptor cells of the primate retina [13]. Although the cell bodies of the photoreceptor cells have minimal expression of the TfR, these structures do express the insulin receptor [13,15]. The differential expression of the TfR and insulin receptor in the ONL may account for the selective expression of exogenous genes in the photoreceptor cells following delivery to the eye with targeting ligands that bind to either the insulin receptor or the TfR. These findings indicate the expression of an exogenous gene in a target organ is a function of both the (a) receptor specificity of the targeting ligand, and (b) tissue specificity of the promoter regulating expression of the trans-gene. If the tissue does not express either the targeted receptor or trans-acting factors that bind to the gene promoter, then there is minimal expression of the trans-gene in tissues following intravenous administration in adult animals.

The lacZ gene under the influence of the bovine rhodopsin promoter is highly expressed in the iris and ciliary body of the adult mouse eye, as well as the corneal endothelium and lens capsule (Figure 4A). These findings parallel observations made in transgenic mice expressing the lacZ gene under the influence of the bovine rhodopsin promoter, where gene expression in the iris and ciliary body was observed [8]. The iris or ciliary body is embryologically related to the photoreceptor cells of the retina. Transfection of iris and ciliary body with the Crx homeobox gene expressed in photoreceptor cells results in synthesis of rhodopsin in the non-photoreceptor structures of the eye [16]. The expression of the lacZ gene under the influence of the rhodopsin promoter in multiple structures of the eye is consistent with prior work showing the TfR is widely expressed in multiple structures of the eye, including the iris, ciliary body, corneal endothelium, the conjunctival epithelium [17], as well as the RPE, the retinal endothelium, and cells of the inner retina [14]. The TfR is also expressed in cells of the choroid [14], although the TfR expression on endothelium of choroidal capillaries is less than the expression of the TfR on the endothelium of retinal capillaries [18]. The choroidal expression of the TfR is consistent with the detection of lacZ gene expression the choroid layer of the eye (Figure 4E).

In summary, these studies indicate that the opsin promoter, like the GFAP promoter [2], enables tissue-specific gene expression in the CNS of the mouse, and eliminates ectopic expression of the trans-gene in peripheral tissues such as liver or spleen that also express the targeted receptor such as the TfR [3]. The non-viral plasmid DNA is expressed episomally and gene expression is transient, although gene expression in the primate eye is still in the therapeutic range for at least 2 weeks after a single intravenous injection of the gene [13]. While the opsin or GFAP promoters may enable CNS-specific gene expression, these promoters have relatively broad profiles of expression within the CNS, and the exogenous gene is expressed in both cerebral and ocular structures. The discovery of gene promoters that are expressed at a more regional level within the CNS will be necessary to achieve regional expression of exogenous genes within specific structures of the brain or eye.

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